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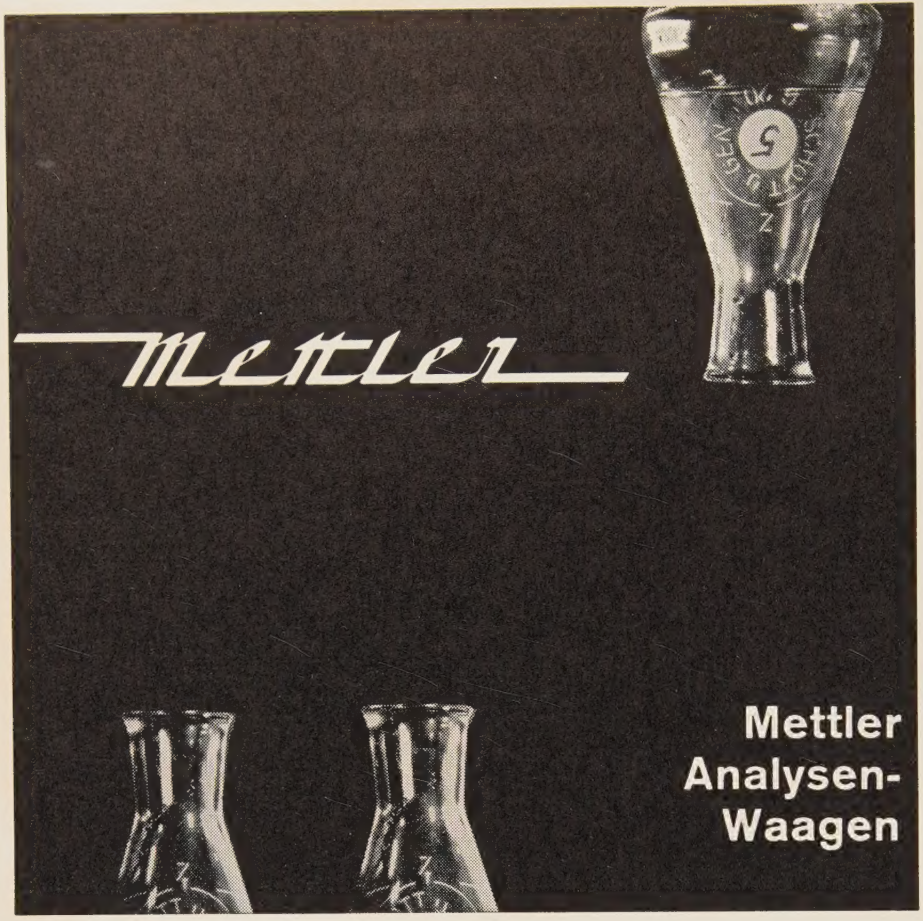

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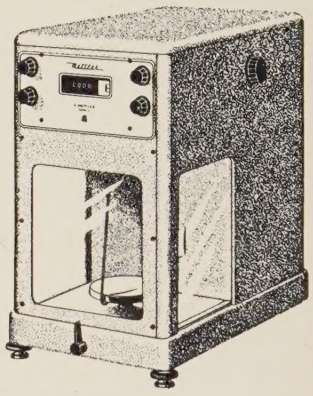

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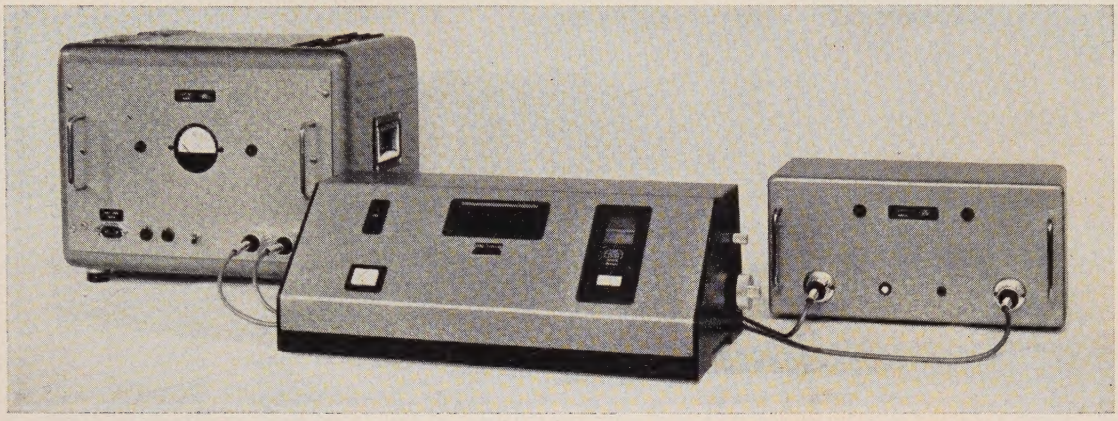

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Density Gradient Electrophoresis as a New Tool in Virology*

By R. CRAMER** and H. SVENSSON***

Introduction. Viruses in the native state are usually present in extremely low weight concentrations, together with large amounts of foreign material, mostly proteins. In crude preparations, it is thus in general impossible to detect or measure the virus by physical or chemical means. The purification procedure, therefore, has to be controlled by infectivity assays, which have a sentitivity 10^4 to 10^8 times as high as, for instance, analysis by ultraviolet light absorption.

Exceptions to the above are some plant viruses, for which there is strong evidence that they have been visualized in crude extracts by physical methods[1]. However, this has not been proved by biological tests.

The isolation of trace amounts of virus from huge quantities of inactive contaminants offers, of course, great difficulties, especially as many viruses are extremely unstable and easily lose their activity under the influence of physical and chemical treatment. The fact that viruses are the very heaviest molecules hitherto known, with molecular weights in the range 10^8, together with the availability during the last few decades of preparative high-speed centrifuges, has greatly facilitated this task. The technique of high-speed differential centrifugation has been extremely important for purification of viruses. In general, microscopic and larger particles of tissue debris are first thrown down by a centrifugation at a comparatively low speed and discarded. The virus is then brought to sediment completely in an extended run at high speed, leaving ordinary proteinous material in the supernatant. The procedure is repeated. With reference to the title of this article, it is interesting to note the increasing importance of high-speed *zonal* centrifugation in density gradients made up by a radially increasing sucrose concentration in the centrifuge tubes. This technique was introduced by BRAKKE[2,3] and was used by him for purification of plant viruses.

Other principles that have been important for purification of viruses are those common to ordinary protein chemistry: salting out with ammonium sulphate, isoelectric precipitation, precipitation by other agents, adsorption and elution. Chromatography on ion exchangers has been used occasionally[4].

Concerning the physico-chemical characterization of purified viruses, especially of preparations that can be regarded as almost 100% pure, sedimentation, diffusion, and electron microscopy are the most important methods. They give together exact information of size, shape and molecular weight of the particles. Light scattering is another useful aid for estimating molecular data[5].

Stability characteristics are of great interest. Like other biologically active substances, the viruses are inactivated by exposure to extreme pH, to various chemical agents, to physical treatment, or simply by storage. Such characterization does not require purified preparations, but they do require infectivity tests.

Electrochemically, a virus is best characterized by its isoelectric point (which may depend on the salt concentration) and by a curve relating its charge, its proton-binding capacity, its electro-kinetic potential, or its mobility with pH. The protonbinding capacity can only be obtained by a direct potentiometric titration of a pure virus sample. The isoelectric point and the pH-mobility curve can be obtained by a series of electrophoresis experiments with preparations sufficiently pure to make it possible to identify a certain peak in the electrophoresis diagram with the active virus. Rough estimates of mobility data may be obtained from crude extracts by conventional electrophoretic methods combined with infectivity titrations.

* The authors express their gratitude to Professor S. GARD (Karolinska Institutet, Stockholm), Dr. A. POLSON (Medical School, Cape Town), and Dr. W. BERNHARD (Institut du Cancer Gustave Roussy, Villejuif) for helpful criticism.

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** Laboratoire Pasteur Institut du Radium, Paris.

*** Department of Bacteriology, Karolinska Institutet, Stockholm (Sweden).

1 V. L. FRAMPTON and W. M. TAKAHASHI, Phytopathology *36*, 129 (1946).

2 M. K. BRAKKE, J. Amer. chem. Soc. *73*, 1847 (1951).

3 M. K. BRAKKE, Arch. Biochem. Biophys. *45*, 275 (1953).

4 J. R. SHAINOFF and M. A. LAUFFER, Arch. Biochem. Biophys. *64*, 315 (1956).

5 G. OSTER, P. M. DOTY, and B. H. ZIMM, J. Amer. chem. Soc. *69*, 1193 (1947).

Electrophoretic methods in virus research. Smaller viruses consist of nucleic acid with a protein coat. Some viruses also contain lipid, but in all probability their surface is mainly, if not entirely, made up of protein. Consequently, the advantages of electrophoresis in the study of proteins should persist for viruses as well.

Compared to other branches of protein chemistry, virology has hitherto gained remarkably little from applications of electrophoretic methods. This is true for purification as well as for characterization of viruses. It seems that this can be partly explained by the fact that electrophoretic methods are incapable of yielding any molecular data, while electrochemical data have not aroused the same interest. Furthermore, hitherto used electrophoretic methods are not ideally suited to work with viruses.

Potentialities of electrophoretic methods in general. Like centrifugation, electrophoresis is an extremely mild method for purification, much to be preferred to precipitating agents, organic solvents etc. Its independence of molecular size and shape makes it a wonderful complement to differential sedimentation, because a preparation with well-defined ultracentrifugal characteristics may well display a whole spectrum of components with different charge characteristics. Thus a preparation of tobacco mosaic virus that appeared homogeneous in the ultracentrifuge was shown by SINGER et al.[6] to represent distinct molecular species when analyzed by electrophoresis.

The electrophoretic method of characterization is very sensitive to small differences or changes in a protein molecule, provided that they affect the net charge of the particle within its stability pH range. Thus hemoglobin S (from sickle cells) can be distinguished from normal hemoglobin electrophoretically at pH 7, although it is known that these two molecular species differ by one single amino acid in a subunit of the molecule, resulting in a difference of 2 charges per molecule. This corresponds, for hemoglobin, to a shift in the isoelectric point of 0.2 pH units. The precision of a measurement of isoelectric point is better than that, about 0.1 pH unit.

In the virus field, mutants can sometimes be differentiated by electrophoretic means. Thus MAC DONALD et al.[7] showed that a spontaneous mutant of southern bean mosaic virus had a pH-mobility curve different from that of the parent strain. GINOZA and ATKINSON[8] investigated the isoelectric points and pH-mobility curves of eight strains of tobacco mosaic virus and could thereby confirm an earlier classification into four groups. A systematic electrophoretic study of viruses might furnish new criteria for their classification.

Possible differences in the electrophoretic mobility of biologically closely related viruses, such as the oncogenic viruses of mice or chickens, might prove to be of great value. In experimental medicine, it is sometimes highly desirable to separate viruses in tissue culture fluid or tumor extracts. For example, on injection of extracts of leukemic tissues of mice and chickens, non-leukemic tumours have sometimes been obtained as well as leukemias. The presence of two distinct viruses has been proved in mice (GROSS[9]; STEWART[10]), but so far not in chickens. Electrophoresis or chromatography might be able to establish whether in chickens one virus causes the two different tumours, or if different viruses are involved.

Another important application of electrophoresis consists in the correlation of various biological properties of a virus, such as infectivity, interference, hemagglutination, complement fixation, and immunoprecipitation, with one or more electrophoretic particle populations. Many other possible applications are concerned with changes occurring when viruses are treated with formaldehyde (FISCHER and LAUFFER[11]), alkali (SCHRAMM et al.[12]), and other chemicals.

Microscopic electrophoresis[13] is based on direct microscopic observation of migrating carrier particles of quartz or collodion on which the protein to be studied has been adsorbed. Such particles acquire the same mobility as the coating protein has in solution. However, the material to be measured has to be very pure, otherwise the carrier particles may be partly or preferentially coated by the impurities. If this condition is satisfied, the method lends itself excellently to determination of isoelectric points and pH-mobility curves of viruses, since the accuracy of these measurements is satisfactory and the amounts of pure virus required are extremely small. Another important advantage of the method is the possibility of measuring mobilities of microscopically visible aggregates of viruses insoluble around the isoelectric point; sorption on carrier particles is then superfluous. Applications of this technique have been presented by OSTER[14], MILLER et al.[15], and SMADEL et al.[16]. A recent review of the method and its applications has been given by BRINTON and LAUFFER[17]. It is not suitable for preparative purposes.

[6] S. J. SINGER, J. C. BALD, S. G. WILDMAN, and R. D. OWEN, Science *114*, 463 (1951).
[7] E. MACDONALD, W. C. PRICE, and M. A. LAUFFER, Arch. Biochem. *24*, 114 (1949).
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[16] J. E. SMADEL, E. G. PICKELS, and T. SHEDLOVSKY, J. exp. Med. *72*, 523 (1940).
[17] C. C. BRINTON, JR., and M. A. LAUFFER, *Electrophoresis, Theory, Methods, and Applications* (Ed. M. BIER, Academic Press, New York 1959), p. 427.

Moving boundary electrophoresis is well suited to electrochemical characterization of pure or almost pure virus preparations. With the best instruments available, mobility values accurate to 10^{-6} cm²/volt sec and better are easily obtained, and, as already mentioned, isoelectric points can be determined to less than 0.1 pH unit provided the virus is sufficiently soluble and can be identified with certainty with an optically visible component.

Studies of this kind have been performed by many investigators. However, many of them have only measured the mobility at a single pH and salt concentration, which is useful for identification purposes but very incomplete as a characterization. BRINTON and LAUFFER[17] have presented a list of hitherto known isoelectric points of viruses; they all lie in the range between pH 3.5 and 6.

The moving boundary method requires comparatively much purified virus material. In the big commercial instruments, about 20 mg is needed for a disturbance-free electrophoretic run, but the material can be saved and re-used in several runs or for other purposes. Nevertheless, this quantity of pure virus is in many cases prohibitively large. In table-size instruments, the quantity required is less, being of the order of 2–5 mg.

Attempts to carry out electrophoresis experiments by Tiselius' method at very low concentrations of pure virus can lead to serious difficulties, and one has to be careful not to lose the costly material (SHEDLOVSKY and SMADEL[18]). The electric current tends to develop irregular thermal convection and laminar electro-osmotic streamings in the electrophoresis channel. These effects actually take place whereever no hydrodynamically stabilizing effect is active. In moving boundary electrophoresis, the only stabilizing effect available is the density gradient accompanying the moving boundaries. Consequently, at a given electric load, there is a minimum concentration of the migrant that can be used. For elementary bodies of vaccinia, SHEDLOVSKY and SMADEL found this critical concentration to be as high as 0.2% by weight, the limiting factor being electro-osmotic streamings, not thermal convection. In the presence of protein with about the same mobility as the elementary bodies, the disturbance was found to disappear. The weak virus boundary was then protected by the additional density gradient given by the protein.

Several authors have reported comparative electrophoretic studies of extracts from diseased and healthy plants or organs (FRAMPTON and TAKAHASHI[1]; TAYLOR et al.[19]). Even if distinct and reproducible differences are found, which possibly can be used for diagnosis, it is always risky to draw conclusions regarding the electrophoretic behaviour of the virus itself from such investigations. A sharp (slowly diffusing) peak in the diagram of the pathologic extract may or may not be the virus. It may also be one of several virus components. No conclusion is safe without infectivity assays on electrophoretic fractions. In preparations not extensively purified, the virus concentration is far below the resolution limit of the best optical methods. Infectivity assays are then necessary to get mobility values.

Such measurements on viruses in the Tiselius apparatus have been described by BOURDILLON and LENNETTE[20], BOURDILLON[21], HARTMAN and LAUFFER[22], and RANDRUP[23]. The crucial difficulty rests in the technique for taking fractions for biological tests. Special sampling devices for the Tiselius apparatus have been described by BOURDILLON and LENNETTE[20], HARTMANN and LAUFFER[22], and RANDRUP[24]. In the latter paper, the sampling accuracy is investigated in detail, and it is shown that the mobility of a virus can be measured to within 10% despite the fact that the relative accuracy of the titration methods is much lower. By keeping the virus boundary stationary in the U-tube by a controlled back-flow of buffer, the electrophoretic run can be much prolonged; the accuracy in the mobility measurement then increases accordingly.

Some difficulties in the use of a Tiselius apparatus for virus analysis by sampling and infectivity titration will be discussed here. The oblong form of the cuvettes, so advantageous for optical analysis, is not very suitable for taking samples through a capillary. The liquid close to the front and rear walls has to flow horizontally a distance of 12 mm before it reaches the capillary tip. Thus an appreciable density gradient is necessary for a successful sampling, and the purer the virus, the less accurate becomes the sampling. The strong density gradients, used by RANDRUP[24] in his assessment of the sampling accuracy, are in general not available in actual experiments, at least not when purified preparations are concerned.

Optical analysis is only of secondary interest in virus analysis by infectivity titration of fractions. To improve the flow characteristics round a sampling capillary, therefore, it would be preferable to use a U-tube with a cylindrical cross-section. This was the usual procedure before the break-through of Tiselius' method (see for instance TODD[25]) and was suggested more recently by POLSON[26], yet without much consequence for virus electrophoresis methodology.

Although RANDRUP's technique of removing the sampling needle for each fraction seems cumbersome

[18] T. SHEDLOVSKY and J. E. SMADEL, J. exp. Med. *72*, 511 (1940).
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[26] A. POLSON, Nature *145*, 27 (1940).

and liable to cause local convection, it is probably about the best that can be done in a standard Tiselius apparatus. Modified U-tubes with fixed capillary sampling tubes at the side of the limbs have been described by SVENSSON[27,28] and KEKWICK et al.[29], but have not been applied to viruses. Undoubtedly, such equipment would allow a much more complete mapping of virus activity versus electric mobility than a standard U-tube. However, the possibilities of detecting two or more virus components by the moving boundary method are nevertheless rather restricted, for the following reasons.

In the term 'moving boundary electrophoresis' lies the fact that the result of the migration is a separation of front and rear *boundaries* only, while the *zones* containing the various constituents still overlap (Fig. 1). Let us now suppose that a certain virus preparation contains two infective components of different mobilities, and that their relative concentrations, in infectivity units, are such that the fastest component can be regarded as the main, the slowest one as a minor constituent. Going from top to bottom in the ascending limb, then, the infectivity will be expected to rise from zero to a high level at the fastest virus boundary, and then to rise a little more at the second boundary. However, *the second, small rise cannot be detected experimentally* because of the rather low relative accuracy of virus titration methods. The standard deviation of such measurements is actually so great that it is difficult to ascertain changes in infectivity smaller than $\pm$ 50%.

The two components can, however, be detected in the other limb, because there the boundary of the minor component comes first. The ultra-high sensitivity of the biological assays secures the detection of the minor constituent, while the rise in infectivity at the boundary of the main component is, according to our assumptions, well over 50%.

To sum up, moving boundary electrophoresis in conjunction with infectivity titration of fractions is capable of demonstrating the presence of two infective electrophoretic fractions in one limb, provided that the number of fractions titrated is great enough, but never in both limbs. If the components are of about the same titer, there is a risk that the second one remains undetected in both limbs. Only the levels at which infectivity first appears may then give an indication of the presence of more than one infective component. In the case of three such components, similar considerations lead to the conclusion that one or two components will escape detection in at least one limb of the U-tube.

As far as known to the authors, nobody has ever titrated a sufficient number of fractions from a moving boundary experiment for proving the electrophoretic homogeneity of a virus preparation.

A certain purification, as well as separation of virus components, can be achieved by the moving boundary method. However, since the material in the bend of the U-tube (Fig. 1) always retains its original composition, the method is not very effective.

Fig. 1 and 2. Separation of two virus components in moving boundary (1) and in zone (2) electrophoresis.

a) Distribution before electro-phoresis; b) Distribution after electrophoresis. Virus component indicated by dots has a higher mobility than component indicated by lines.

Zonal electrophoresis methods. The limitations of the moving boundary method discussed above have caused difficulties not only in virus research, but in other applications as well. During the last decade, therefore, there has been an extensive development of various methods for *zonal* electrophoretic separation. The principle, illustrated in Figure 2, consists in having a conducting channel stabilized against convection, in which a narrow zone of the sample is introduced. Under the influence of the current, the various components are split up into discrete zones, completely separated if a sufficient time is allowed.

Considering again the case of two electrophoretic virus components, it is easily realized that both can be detected and localized, whatever their relative concentrations are. Provided that the constituents are well separated and that a sufficient number of fractions are titrated, the bad relative accuracy of virus titrations does not come into play, while use is made of the high sensitivity. Zonal electrophoresis is thus ideally suited to virus analysis.

According to the method used for hydrodynamic stabilization, these methods can be subdivided into paper electrophoresis, gel electrophoresis, electrophoresis in powder (starch, cellulose) columns or slabs, density gradient electrophoresis, liquid film electrophoresis, laminar flow electrophoresis, and rotating tube electrophoresis. The three last versions have been very little developed and will not be discussed here. Some view-points regarding the application of the others to virology will be given below.

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[28] H. SVENSSON, Ark. Kemi Min. Geol. *15B*, No. 19 (1942).
[29] R. A. KEKWICK, J. W. LYTTLETON, E. BREWER, and E. S. DREBLOW, Biochem. J. *49*, 253 (1951).

Paper electrophoresis has grown to an immense importance in protein chemistry during the last five years. In view of the copious literature in this field, it is very remarkable that almost nothing has been published on paper electrophoresis of viruses. Probably many investigators have tried, but met with difficulties or got inconclusive results which have not been published. Extensive sorption to the paper fibers is the probable cause of such difficulties. Sorption gives rise to tailing of migrating zones and to difficulties in obtaining complete elution of virus from the paper.

That sorption of virus to paper is a reality and not only a hypothesis is shown very nicely in a paper by ZAITLIN[30], although his application of paper electrophoresis was completely successful. He used continuous electrophoresis in a vertical paper curtain and demonstrated that two strains of tobacco mosaic virus could be completely separated. However, the relative amounts of the two strains collected from the curtain did not correspond to those fed to the starting point. On the other hand, when the experiment was interrupted and the curtain dried and stained for protein, the relative amounts of bound dyestuff was wrong in the other direction. This proves that one of the strains was sorbed to the paper much more strongly than the other. In the continuous technique, a reversible sorption effect does not impair the separation and purification, because the sorption influences the vertical flow as much as the horizontal migration. Sorbed or free, all molecules then stick to the same migration path through the curtain. Quantitative analysis, however, is only possible after complete elution of the virus from the paper by allowing buffer and current to flow for a considerable time after the flow of virus suspension has been stopped.

Electrophoresis in gels and powders can be expected to offer the same difficulties when applied to viruses. Even here the information available in the literature is very scanty. HIRTZ and CAMAND[31] have investigated the electrophoretic behaviour of foot-and-mouth disease virus in a starch trough, the migration being analyzed by measurement of complement fixation. They found at least two complement-fixing components in each strain, and no complications due to sorption are apparent from their report.

Density gradient electrophoresis. In this technique, hydrodynamic stability is obtained in a vertical column of free liquid by preparing the latter in such a way that the density steadily increases from top to bottom. Thus the column is characterized by a density gradient, which effectively opposes the current's tendency to develop thermal convection and electroosmotic streaming. The increasing density is generally obtained by arranging for an increasing concentration of sucrose.

Sorption and molecular-sieving effects are completely absent in this method. In contrast to the moving boundary method, there is no lower limit to the concentration of the sample to be analyzed. As a matter of fact, the method works better and safer the lower the concentration is.

Since the density gradient stabilizes the liquid even after the run, no stop-cocks or other mechanical means for subdividing the column into compartments are necessary. The contents can simply be emptied slowly through a stationary capillary, from the other end of which any number of fractions can be taken. The curve relating virus activity with the level in the column can thus be obtained in corresponding detail. Because of the complete separation into discrete zones, enough material can be obtained for testing different biological properties of the virus in each fraction. The electrophoretic resolution of components is good. The removal of the virus from the column is practically quantitative, the only possible losses being due to a slight adsorption to the walls of the column and the sampling capillary.

Mobility values can only be obtained by inclusion of (preferably coloured) reference substances with known mobilities. This limitation the method shares with those using solid support. Hemoglobin and bromophenol-blue-stained serum albumin can be recommended as reference substances. Since serum albumin is the only component of serum that combines with bromo-phenol-blue in the native state, the practical procedure is to add hemolyzed serum and a very small quantity of bromo-phenol-blue to the virus sample. The addition of two such reference substances allows the construction of a mobility spectrum of virus activity with a fair accuracy.

The experimental facts and the considerations based thereon which have been reviewed on the preceding pages reveal without doubt that electrophoresis in general is capable of rendering much more service to virology than has been realized hitherto. This statement refers to purification as well as to electrochemical characterization of viruses. Moreover, it is evident that, at the present state of development of electrophoretic methods, the density gradient technique offers quite special advantages to virus research that cannot be achieved by any other electrophoretic procedure in current use. For this reason, a survey of the results gained hitherto, during a couple of years only, in virus research by density gradient electrophoresis will be presented in the next section.

Experiment techniques for density gradient electrophoresis. Various methods and apparatus for density gradient electrophoresis have been reviewed recently by SVENSSON[32]. A short description of the technique

[30] M. ZAITLIN, Biochim. biophys. Acta *20*, 556 (1956).

[31] J. HIRTZ and R. CAMAND, Rev. Immunol. *18*, 206 (1954).

[32] H. SVENSSON, *A Laboratory Manual of Analytical Methods in Protein Chemistry, including Polypeptides* (Eds. P. ALEXANDER and R. J. BLOCK, Pergamon Press Ltd., London 1960), Vol. 1, p. 193.

used for work with viruses by CRAMER et al.[33–36] is given below.

The apparatus used in these investigations is shown in Figure 3. An U-tube provided with a wide stop-cock in one limb is connected to two electrode vessels with capillaries at their lower ends. One limb of the U-tube has a vertical extension which is closed by a rubber stopper through which passes a capillary sampling tube A and a drainage tube B. Sometimes two sampling capillaries were used. The electrodes are silver wires coated by silver chloride and are always surrounded by a concentrated chloride solution.

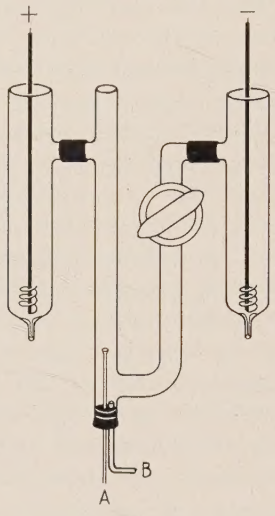

Fig. 3. Electrophoresis apparatus

The apparatus is first filled with buffer solution to a level slightly above the tubes connecting the U-tube with the electrode vessels. A solution of the same buffer composition and containing in addition 40% sucrose is then introduced through tube B and is allowed to rise to the level of the tip of tube A. The stop-cock is then closed, and the positions of the menisci are marked outside the electrode vessels.

In the gradient-mixing device shown in Figure 4, the flask P_1 is filled with buffer without sucrose, and P_2 with heavy buffer containing 40 % sugar. The device is placed at a level higher than the menisci in the apparatus, and the outlet tube A is connected to the capillary tube A in Figure 3. After a magnetic stirrer in flask P_1 has been started, the contents of the mixer are allowed to run into the apparatus slowly. The mixer delivers a solution with a steadily increasing sucrose concentration, which displaces the light buffer in the apparatus towards the left-hand electrode vessel. The level of the meniscus is readjusted to the mark.

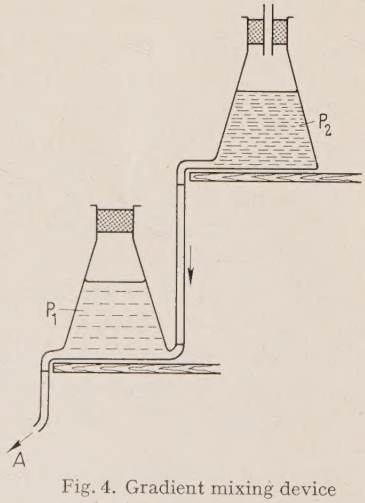

Fig. 4. Gradient mixing device made of two Erlenmeyer flasks, P_1 containing buffer, and P_2 containing 40% sucrose in the same buffer.

Saturated sodium chloride solution is next pressed into the electrode vessels until the electrodes become completely covered by it. After the menisci have again been readjusted to the marks, the stop-cock is cautiously opened. Because of the increased hydrostatic pressure in the left-hand limb, the menisci will now assume a small level difference, and the original boundary between heavy and light buffer will be shifted somewhat downwards.

The apparatus is now ready for receiving the virus sample, but before that one has to be sure that the latter has a suitable density. To that end, two Vidal tubes are prepared, one containing the heavy buffer solution with 40% sucrose, the other a sample from the column obtained by temporarily opening tube A in Figure 3. Sucrose is dissolved in the virus suspension to give approximately 35%. The sample has a suitable density if one droplet thereof floats on the surface in the first Vidal tube and sinks to the bottom in the other. The sucrose concentration has to be adjusted up or down until this condition is satisfied. The virus sample can then be pressed into the column through the capillary tube A.

After about 20 min, diffusion of sucrose across the initial virus zone has created a density gradient within it strong enough for starting the current. The duration of the electrophoretic run depends on the virus material. Short runs of only 4 h have been performed with very unstable viruses, whereas in general the duration has been extended to about 16 h, with a current of 15 mA and a potential gradient of 3.5 V/cm. A cooling system adapted to the electrophoresis apparatus has been constructed, allowing work at a constant temperature of 4° C and less. Substances stabilizing the biological activities of viruses can be added to the whole column. Rabbit hemoglobin and phenol red, when added to the virus suspension in some experiments, were useful guides to the progress of the migration.

After electrophoresis the stop-cock is closed, and the column is emptied through capillary tube A and subdivided into fractions. Since traces of virus may stick to the wall of this capillary from the injection procedure before the run, it may be advisable to use two capillary tubes, one for injection of the initial zone, the other for sampling.

In order to describe more fully the experimental procedure, a specific example of an experiment with poliomyelitis virus will also be related. This virus is relatively thermoresistent.

Poliomyelitis tissue culture fluid was concentrated by pervaporation (POLSON and HAMPTON[37]) and purified by three cycles of differential ultracentrifugation.

[33] R. CRAMER, K. D. LERNER, and A. POLSON, Science Tools *4*, 17 (1957).
[34] A. POLSON and R. CRAMER, Biochim. biophys. Acta *29*, 187 (1958).
[35] R. CRAMER, Nature *183*, 195 (1959).
[36] R. CRAMER and S. STEWART, Proc. Soc. exp. Biol. Med., N. Y. *103*, 697 (1960).
[37] A. POLSON and J. W. F. HAMPTON, J. Hyg., Camb. *55*, 344 (1957).

To 0.3 ml of this concentrate with a titer of $10^{7.85}$ ID 50 per 0.1 ml, 0.7 ml of a 35% sucrose solution in buffer and one drop of rabbit hemoglobin was added. The material was introduced carefully by a syringe through the capillary A, Figure 3. Due to the red colour, the outflow of the virus-containing material above the tip of the capillary was easily controlled. After the virus sample, heavy buffer solution was injected through the same capillary till the virus material had left the capillary and formed a flat layer 1–2 mm thick between the 40% sugar level below and the sugar gradient above. 20 min later electrophoresis was started and allowed to proceed for 16 h at room temperature. Then the stop-cock was closed and the column fractionated into samples of 0.5 ml each, using a clean capillary as recommended above. The virus activities of the samples were determined by injection of 10-fold dilutions into groups of 5 tubes of trypsinized monkey kidney cells. Each tube received 0.1 ml. The 50% infectivity end-points were calculated by the method of Reed and Muench[38]. The infectivity titers found for each sample are indicated in Figure 5. The amount injected into the column was 8.32 log TCID 50, while that recovered was 8.09 log TCID 50. The recovery of virus activity was thus about 58% in this experiment.

Review of results obtained by the density gradient method. Brakke[39] was the first to use density gradient electrophoresis for viruses. Cramer, Lerner, and Polson[33] applied the method to the study of the FA strain of mouse encephalomyelitis virus. So far the Stockholm and Cincinnati strains of poliomyelitis virus type I (Polson and Cramer[34]), the Rous sarcoma virus (Cramer[35]), and a hemagglutinating as well as a masked strain of polyoma virus (Cramer and Stewart[36]) have been examined by the same method. Some results will be discussed here in order to demonstrate the value of the method for work with viruses. The main purpose of these studies was to gain possible new criteria for their *classification by determining their electrophoretic mobilities*. For example, Theiler's virus, which is very closely related to the group of poliomyelitis viruses, exhibits an entirely different mobility. Polson (personal communication) has succeeded in an electrophoretic classification of the ECHO, poliomyelitis, and Coxsackie virus groups. A similar study of the cancer viruses has been started by one of us (Cramer). The viruses are being characterized by their relative mobilities, that is, their migration distances compared with those of added reference substances, such as rabbit hemoglobin or neutral red; their mobilities have been accurately measured in the Tiselius apparatus.

A phenomenon that has to be discussed in some detail is the *electrophoretic inhomogeneity* found for some viruses, e.g. poliomyelitis (Fig. 5) and Rous sarcoma virus (Fig. 6). Such results were already suspected on the basis of inhomogeneity found earlier for some viruses when analyzed by other methods, such as immunoprecipitation (Le Bouvier, Schwerdt, and Schaffer[40]; Polson, Ehrenberg, and Cramer[41]) or analytical centrifugation (Polson, Ehrenberg, and Cramer[42]). The possibility that the electrophoretic inhomogeneity might be due to an artefact was very carefully considered. On the one hand, an artefact due to the sampling technique was excluded by the following experiment. Poliomyelitis virus was introduced into the electrophoretic column and displaced to a position about 6 cm above the capillary tip in Figure 3.

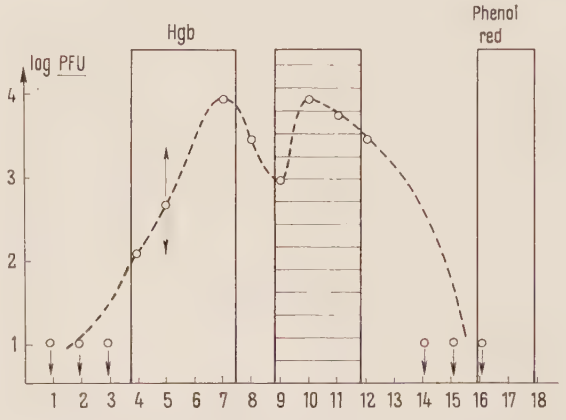

Fig. 5. Zone electrophoresis diagram showing the distribution of Rous sarcoma virus activity in log plaque-forming units (PFU) for each consecutive electrophoretic fraction of 0.8 ml each, indicated on the abscissae. The positions of the reference substances are indicated by the first and third vertical columns and the opalescent zone containing the impurities by the second vertical column. [Reproduction from Cramer, Nature *183*, 195 (1959).]

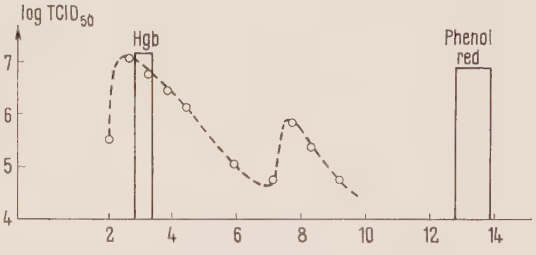

Fig. 6. Electrophoresis diagram of purified Stockholm type I poliomyelitis virus. The vertical columns indicate the positions of the reference substances. The virus titers are given in log TCID-50 units on the ordinate for each consecutive fraction of 0.5 ml, fraction numbers being along the abscissae. [Reproduced from Polson and Cramer, Biochim. biophys. Acta *29*, 189 (1958), with the permission of the journal.]

[38] L. J. Reed and H. Muench, Amer. J. Hyg. *27*, 493 (1938).
[39] M. K. Brakke, J. Amer. chem. Soc. *73*, 1847 (1951); Arch. Biochem. Biophys. *45*, 275 (1953).
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[41] A. Polson, A. Ehrenberg, and R. Cramer, Biochim. biophys. Acta *29*, 622 (1958).
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When the virus was sampled from this position, only one peak of virus activity was found. On the other hand, the development of an artefact during electrophoresis due to convection currents before sampling does not seem to occur, for two reasons. First, when the fast-moving fraction of poliomyelitis virus was separated from the slow one and subjected to a repeated run, it exhibited the same mobility, and no slow activity peak was found. Second, certain viruses were found consistently to be electrophoretically homogeneous, that is, to have a single mobility, which again excludes an artefact as the factor underlying the electrophoretic inhomogeneity found for other viruses. It seems, therefore, that the assumption that highly purified virus populations must always be homogeneous when tested by various physical methods should be reconsidered. The finding of inhomogeneous populations is attributable to the application of improved analytical methods.

Correlation of the *electrophoretic mobility* of a virus with its *degree or mode of purification* hast just begun. Identical electrophoretic diagrams were recorded for native and highly purified poliomyelitis virus type I. For this virus, therefore, no measurable changes in mobility due to removal of impurities or due to changes attributable to the purification procedure seem to occur. On the other hand, slightly different diagrams were obtained for Theiler's virus when different purification methods, such as the ether and chloroform methods, were used. No other comparative data on viruses purified by different methods are known to the present authors. However, it might be of value to study such effects systematically.

It is not yet clear how the finding of apparently distinct electrophoretic populations for a virus of biologically well-defined specificity, as has been found for poliomyelitis virus type I (POLSON and CRAMER[34]) and Rous' sarcoma virus (CRAMER[35]), is to be interpreted. The biological specificity of the former virus is demonstrated by the fact that, for both the Stockholm and the Cincinnati strain, the tissue culture supernatant used for the electrophoretic experiments exhibited a cytopathogenic effect in tissue culture specifically neutralized by type I poliomyelitis antiserum. Likewise, the tissue culture supernatant used for electrophoresis of Rous' sarcoma virus induced pocks on the chorioallantoic membrane, which is highly specific for Rous' virus, but did not induce these tumours when previously incubated with anti-Rous-sarcoma serum from a chicken with regressing Rous tumour.

The possibility that electrophoretically distinguishable virus fractions may exhibit some difference in biological specificity cannot be excluded.

POLSON and CRAMER[34] found that the relative abundance of the electrophoretically faster and slower components of poliomyelitis virus type I was significantly different between the avirulent Cincinnati and the highly virulent Stockholm strain. This discovery is interesting, but continued investigations including *in vivo* experiments are required before any definite conclusions can be drawn.

Biological properties, probably related to the surface of a virus particle, have been examined, but so far only to a very limited extent. The binding of homologous antibody to the surface of the virus is such a property. Preliminary results (unpublished) for poliomyelitis virus type I do not show any difference in neutralizability between the two electrophoretically distinguishable virus populations. Another immunological method, immunoprecipitation, was used in the case of Rous' sarcoma virus (unpublished). Each electrophoretic fraction was tested in agar by the method of OAKLEY and FULTHORPE[43]. Serum from chickens with regressing Rous tumours and with known neutralizing capacity for Rous' virus was used. As indicated in Figure 7, very faint precipitation lines were recorded, usually only in fractions containing much infective virus. In order to test if the antigens of the two electrophoretic populations were identical

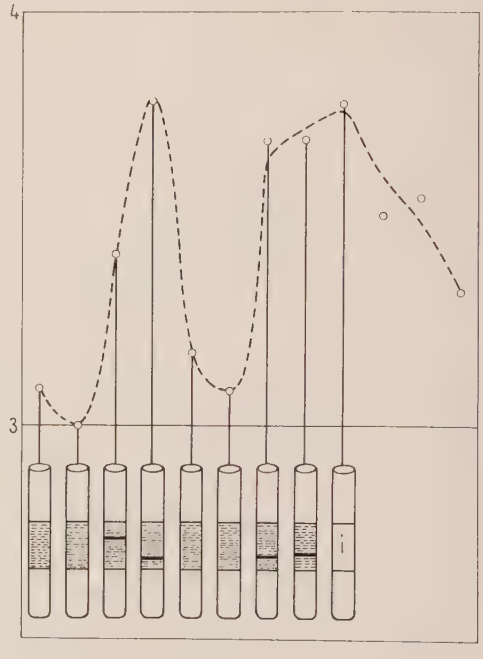

Fig. 7. Zone electrophoresis diagram showing the distribution of Rous sarcoma virus activity in log plaque-forming units for each consecutive electrophoretic fraction as indicated on the abscissae. The tubes show results of immuno-precipitation tests performed for each fraction, according to OAKLEY and FULTHORPE. The middle part of the tube represents a neutral zone of 0.9% agar, resting on a zone containing 0.9% agar mixed with serum of a chicken with regressing Rous tumour and previously determined neutralizing capacity for Rous virus. A sample of each electrophoretic fraction had been layered above the neutral zone. Faint precipitation lines appeared after two weeks with the fractions of highest virus activity.

[43] C. L. OAKLEY and A. J. FULTHORPE, Arch. Biochem. *11*, 337 (1953).

or not, a confrontation of the corresponding antigens by the Ouchterlony technique was attempted, but so far no precipitation lines have been obtained in this system.

A combined electrophoretic and immunologic analysis is already available in the form of the ingenious immunoelectrophoretic method described by GRABAR and WILLIAMS[44]. The applicability of this method to viruses has not been demonstrated; probably sorption and molecular-sieving effects will cause difficulties. In density gradient electrophoresis, such difficulties are entirely out of question. Consequently, it may be more advantageous for virologists to try a combination of density gradient electrophoresis and the immunoprecipitation technique described by OUCHTERLONY[45] and OUDIN[46].

Density gradient electrophoresis has proved to be of value also for the *purification of viruses*. For poliomyelitis virus, it has been found that the active agent separates well from a fastermoving opalescent zone. For Rous' virus, a first peak of virus activity was found to separate from a faster migrating opalescent zone still containing some virus activity. A separation of the active agent from two opalescent zones has been observed for polyoma virus as well. The study of many viruses, especially cancer viruses, is still seriously hampered by the lack of satisfactorily purified virus preparations. The purification effects obtained for unstable viruses, such as Rous' sarcoma virus, which are not resistant to organic solvents, are of special interest.

It is sometimes desirable to remove not only impurities, but also biologically active material, such as *virus inhibitors*, from a virus preparation. In the case of a masked non-hemagglutinating strain of SE polyoma virus, a definite separation of the hemagglutinin from an inhibitor substance has been achieved (CRAMER and STEWART[36]).

From the above considerations, it is evident that electrophoretic methods are capable of rendering a greatly increased service to virus research. Although this statement refers to all electrophoretic methods in current use, the authors are convinced that the density gradient method offers special advantages to workers in the virological field. The viruses, being excessively large molecular entities, are more susceptible to sorption and molecularsieving disturbances than are smaller molecules of biological interest, when electrophoresis in paper, powders, or gels is tried. The density gradient method rules out these secondary influences altogether. Moreover, a maximum of sampling accuracy is ascertained by this method, and no problems concerning quantitative elution of active material are relevant. The fractions obtainable from density gradient runs can be assayed by a multitude of biological tests, which is very essential for work with viruses.

Résumé. Les avantages de la méthode d'électrophorèse en gradient de densité pour des travaux en virologie sont analysés.

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[46] J. OUDIN, Ann. Inst. Pasteur *75*, 30 (1948).

Renal and Extrarenal Actions of Aldosterone*

By F. GROSS**

The isolation, identification, and the synthesis of aldosterone were accomplished in a situation and under conditions quite different from those existing for other adrenocortical hormones. Already before the genuine hormone became known, there was a steroid – cortexone – isolated from the adrenal cortex, the action of which corresponded quite well to the concept of the rôle which the natural hormone would play in sodium metabolism. Some investigators even went so far as to claim cortexone to be the essential secretory product of the adrenal cortex. Hence, when the natural hormone was recognised, it met a biased situation in so far as it was expected to produce the same effects as its predecessor which was already designated as 'sodium retaining' or, even worse, as 'mineralocorticoid'. The fact that, due to the complicated synthesis, only very small quantities of aldosterone were available for several years had the consequence that the majority of investigations of this hormone concern its excretion in the urine and to a lesser degree its secretion rate in various normal and pathological conditions. It is understandable that in view of the characterisation as a sodium retaining hormone, most efforts in this research were directed towards the detection of an elevated aldosterone secretion in pathological conditions associated with a retention of sodium or with edema formation. The syndrome of primary aldosteronism was

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** Forschungslaboratorien der CIBA Aktiengesellschaft, Pharmazeutische Abteilung, Basel. – I should like to express my thanks to Dr. G. T. BASSIL for his help in preparing the English version of the manuscript.

established by CONN[1,2], and in various pathological states which are accompanied by an elevated aldosterone secretion or excretion, the hormone was suspected of participating in pathogenesis. It has, however, to be mentioned that this overemphasis of a possible pathogenic rôle of aldosterone led to a neglect of its physiological functions and the therapeutic importance which it might have under certain conditions. In our opinion, the present characterisation of aldosterone merits some reconsideration, and to this end, the various activities of the hormone will be discussed from the following points of view:

1. The action on the renal transport of sodium. 2. The restorative effects in the adrenalectomised animal. 3. The consequences of prolonged administration of high doses of aldosterone. 4. The extrarenal effects. 5. The antitoxic effects of aldosterone in comparison with other corticoids.

1. *Rôle of Aldosterone in the Tubular Transport of Sodium.* As was shown by PITTS[3], removal of the adrenals diminishes the tubular reabsorption of sodium by only about 2%, indicating that the responsible hormones play but a modifying part in the fundamental process of sodium conservation. By administering aldosterone directly into one renal artery of the normal unanesthetised dog, no definite effect on sodium excretion can be demonstrated, while potassium elimination increases after one hour's infusion. In the adrenalectomised animal, however, a definite diminution of the elevated sodium excretion was obtained, first in the infused and, later on, also in the contralateral kidney, being followed, after a certain delay, by a kaliuresis (BARGER et al.[4]). These results have been confirmed in principle by other investigators who also found a delay in the onset of sodium retention in the adrenalectomised animal, indicating that aldosterone, like cortexone[5], takes time to influence the tubular function, contrary, for example, to the effect of ADH which is already manifest a few minutes after the beginning of an intrarenal infusion[4]. The delayed onset of action of either aldosterone or cortexone cannot be explained on the basis of the data available, but it can only be assumed that it may be due to a slow adaptation of the enzymes involved in the tubular cation exchange[6], or that the change is so small that it needs a cumulative effect in order to become manifest.

Contradictory findings were reported about the site of action of aldosterone in the nephron[7–13], but with the aid of the stop-flow technique, it was possible to decide that the hormone influences sodium absorption mainly in the distal tubules (VANDER et al.[11]). In the adrenalectomised dog, aldosterone restores the capacity of the distal tubules to almost complete reabsorption of sodium, while it does not influence sodium concentration in the proximal tubules[13,14]. Aldosterone also restores the reduced sodium concentration gradient between the tubular cells and the lumen which in the adrenalectomised animal depends to a certain degr on the plasma sodium concentration, while in the pr sence of aldosterone, sodium concentration in the dist tubular urine is lowered, regardless of the plasma s dium concentration (VANDER et al.[13]).

Aldosterone favours tubular potassium and hydri (titratable acid) excretion and differs in this respe from cortisol which only stimulates potassium elimin tion[7,15–18]. This different effect on potassium excreti also becomes evident from investigations on combin administration of corticosterone or cortisol and ald sterone, from which it was found that both hormon together lead to a more pronounced kaliuria th aldosterone alone, whilst its sodium retaining effect diminished[19]. In patients with Addison's disease, i fusion of aldosterone may rather lead to a more pr nounced decrease of sodium excretion than to an i crease in potassium loss[20,21]. Under a low sodium di or in patients with secondary aldosteronism, either d to congestive heart failure or liver cirrhosis, furth administration of aldosterone causes almost comple sodium reabsorption but does not necessarily increa potassium excretion, indicating that sodium is n exclusively reabsorbed by direct exchange against p tassium[8,22]. In this connection, the attractive hyp thesis of VÉSIN[23] has to be mentioned, according which the site where aldosterone influences sodium

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absorption is situated in the collecting duct where ammonium ion is simultaneously secreted, and also in the distal tubules where potassium secretion is favoured. Hence, the mechanism of tubular cation exchange seems to be more complex than supposed so far, but further experiments are necessary to evaluate the modifying rôle aldosterone plays in the conservation of sodium and the maintenance of ionic equilibrium by the kidney.

Antagonism against the Effect of Aldosterone on Sodium Reabsorption in the Kidney. Recently, various steroids of the spirolactone type were found to produce natriuresis without influencing or even lowering the excretion of potassium, differing in this respect from the benzothiazide derivatives which stimulate the elimination of both cations[24, 25]. The spirolactones have only a natriuretic effect in the presence of sodium retaining steroids and act as competitive antagonists to aldosterone at the site in the distal tubule, where the hormone favours the reabsorption of sodium. Simultaneously with the decrease of potassium elimination, the spirolactones also diminish the excretion of titratable acid and of ammonia, confirming by this that aldosterone stimulates the excretion of hydrion as well as of potassium[26, 27]. In rats as well as in man, spirolactones do not necessarily increase aldosterone secretion if a normal diet containing adequate salt is given, but it was found in rats that with a low salt diet, spirolactone markedly stimulates aldosterone secretion[28]. This observation underlines the importance of preexisting conditions in this and other types of experiment on the secretion and function of aldosterone.

Whether progesterone possesses a similar type of competitive antagonism to the sodium conserving effect of aldosterone, and if the natriuretic effect of high doses of progesterone is the consequence of a peripheral inhibition of endogenous aldosterone, remains to be settled[29].

Inhibition of the sodium reabsorption effected by aldosterone has also been reported in the rat for vasopressin and oxytocin, leading to sodium excretion in this animal species[30]. There are, however, no indications that the two polypeptides act as physiological antagonists to aldosterone in controlling renal tubular function.

2. *Restorative Effects in the Adrenalectomised Animal.* In a series of experiments, SWINGLE et al.[31] found that in the adrenalectomised dog showing distinct symptoms of adrenal insufficiency, corticoids, such as prednisone and particularly 2-α-methyl-9α-fluoro-cortisol, were capable of alleviating rapidly the condition of the animal which was kept without food and water, and of restoring in part, but not completely, the plasma electrolyte pattern. Aldosterone, however, like cortexone, revealed itself to be without effect under these conditions, although given repeatedly at intervals of only a few hours in doses as high as 4 mg.

Under similar conditions, we compared prednisolone and aldosterone administered intravenously to adrenal insufficient dogs in single doses of 10 mg (prednisolone in the form of the sodium salt of the tetrahydrophthalate, aldosterone as unesterified racemate), but gave the animals free access to food and water. While prednisolone had an almost immediate effect on the general condition of the animal, which became alert, less atactic and began to eat, a delay of about 6 h occurred before the same effects were apparent in the dogs treated with aldosterone. This delayed response to aldosterone was also evident in the changes in various parameters in the plasma (Fig. 1).

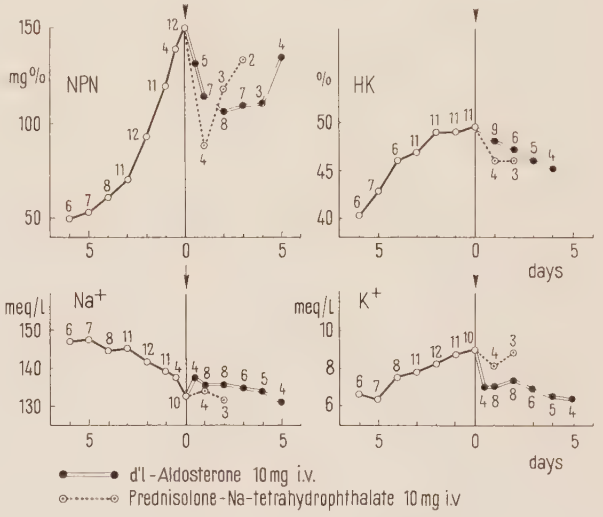

Fig. 1. Effect of a single dose of aldosterone and Prednisolone in the adrenal insufficient dog.

While under prednisolone, the non-protein nitrogen concentration fell markedly within 24 h, it took about twice this time with aldosterone, but the effect lasted also about twice as long as with prednisolone. The general condition of the animals treated with a single dose of aldosterone was definitely improved for 4–5 days, while after prednisolone, the effect lasted only 2–3 days. Neither plasma sodium nor potassium concentration was restored to normal with either hormone, but aldosterone produced the more marked effects, particularly in lowering elevated potassium concentra-

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tion, which even as long as 5 days after the single dose was still definitely lower than at the time of injection. Similar variations were found in the hematocrit values which were also definitely lowered over 5 days by a single aldosterone injection.

It can thus be stated that, by treatment with a single dose of aldosterone, a severe state of adrenal insufficiency in the dog can be partly abolished, but that the hormone acts with a certain delay and less dramatically than prednisolone or similar corticoids. This retarded response observed by us in the dog is in contrast to findings reported by FRIEDMAN[32], who found in the adrenalectomised rat within 30–60 min after the injection of aldosterone not only a return of the low blood pressure to normal values, but also a restoration of the pressor response to pitressin.

Effect of Aldosterone on the Excretion of Water and Salt Load. COLE[33,34] has demonstrated that the intact rat loses sodium in response to infusions of isotonic saline and that aldosterone as well as cortexone in a dosage range which approximately corresponds to normal secretion rates reduce the sodium loss in part but not completely. It remains to be investigated if by larger amounts of aldosterone, this increase of the tubular rejection fraction may be completely abolished.

In earlier experiments, we observed that the adrenalectomised dog under maintenance doses of aldosterone is not able to eliminate a given load of water or salt in the same way as the intact animal[35]. Similar findings have been reported about the effect of aldosterone on water intoxication in the adrenalectomised rat[36] and on salt load in the adrenalectomised dog[37]. Pursuing these investigations, we have now found that by increasing the daily dose of aldosterone to 1000 γ in the form of the racemate, the capacity of the adrenalectomised dog to eliminate a load of salt (1 g/kg) is definitely better than under the influence of the minimum maintenance dose (Fig. 2). For a water load (90 ml/kg), the improvement in excretion as a result of increasing the doses of aldosterone was less marked. This is in contrast to observations with cortexone which, if given in doses of 20–30 mg daily, leads to a more delayed excretion of a given salt load, if compared with the effect of a maintenance dose. A water load, however, is more rapidly eliminated under high doses of cortexone than under the influence of a maintenance dose of this corticoid. These observations led us to

3. *Prolonged Administration of High Doses of Aldosterone.* The scarcity of supplies had the consequence that the effects of exogenous overdosage with aldosterone were not studied to the same extent as the results of endogenous overproduction. On the basis of our own observations of continuous overdosage in the rat[38], in the rabbit[39], and in the dog[40], we may, however, state that the syndrome which develops under high doses of aldosterone and salt is in various respects qualitatively similar to that occurring under high doses of cortexone, although quantitatively definitely le pronounced. In the rabbit, symptoms of increas capillary permeability and of potassium loss preva leading to effusions and to paralysis of skeletal as w as of involuntary muscle (Fig. 3), but not to an i crease of blood pressure[39].

In rats, the average daily secretion rate of aldost rone was found to be 0.4γ/kg/adrenal/h under norm conditions[41]. This corresponds to nearly 20 γ/kg duri 24 h, provided that this secretion rate were to contin during the whole day. An animal of 150 g body weig

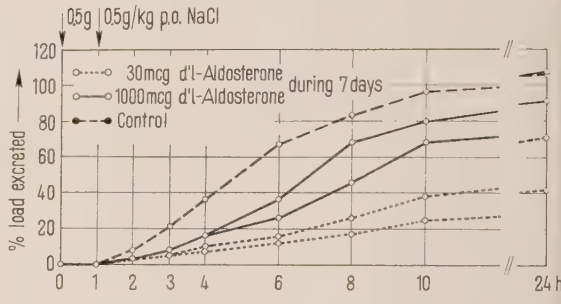

Fig. 2. Effect of a low and a high dose of aldosterone on the excret of a salt load in the adrenalectomized dog. Pretreatment with aldosterone in the doses indicated on the grap days before loading.

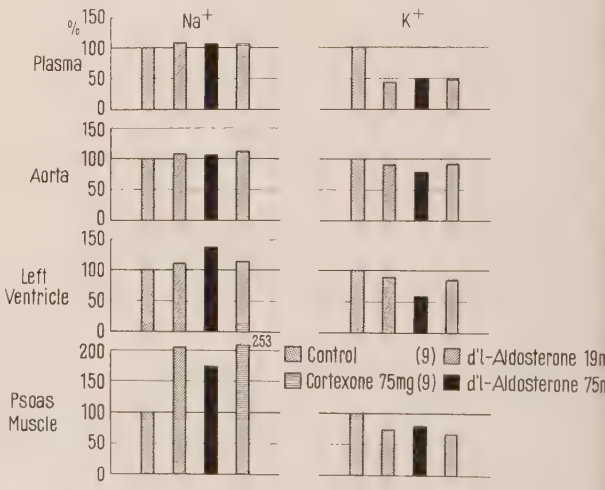

Fig. 3. Overdosage of aldosterone and cortexone in the rab Relative concentration of sodium and of potassium in various tiss under high dosage of aldosterone and of cortexone administered the form of pellets. Data taken from GROSS and SCHMIDT[39].

[32] S. M. FRIEDMAN, C. L. FRIEDMAN, and M. NAKASHIMA, Am J. Physiol. *195*, 621 (1958).
[33] D. F. COLE, J. Endocrinol. *14*, xlii (1957).
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would accordingly secrete about 3–4 γ of aldosterone per day (Table). Considering these figures, one wonders how GORNALL et al.[49, 50] could provoke hypertension and other pathological changes by giving less than the daily amount of endogenously produced aldosterone to intact rats, even if one takes into account the long duration of their experiments, lasting 3–6 months. Hence it is not surprising that these results could not be confirmed by other investigators[51, 52]. In order to provoke hypertension in the rat, in our own experiments, it was necessary to give about 50 times larger amounts than those secreted by the gland, together with free access to saline[38]. It has, moreover, to be stated that within the period of observation, the degree of blood pressure elevation as well as the morphological alterations in kidney, heart, and arteries were definitely less pronounced – if detectable at all – than after giving cortexone in a five times higher dose. In our opinion, it is not justified to draw any conclusion from these experimental results on the rôle which aldosterone may possibly play in the pathogenesis of essential hypertension. The finding of an elevated secretion or excretion of aldosterone in various forms of human hypertension, recently reported, does not give enough evidence for establishing a primary pathogenic rôle for aldosterone, but may be a consequence rather than the cause of this disease (LAUGH et al.[53]).

Secretion rate of aldosterone in γ/kg/day

	normal		low salt intake		high salt intake	
rat	19,7	[41]	27,7	[41]	6,4	[41]
	14,7	[42]				
dog	4,7	[43]	8,5	[43]		
	2,1	[44]				
	5,1 [360]	[45]	8,0	[45]		
human	♀3,2 [192]	[46]	11,0	[46]		
	3,4 [240]	[47]	13,6	[47]	0,7	[47]
	♂1,3 [77	[48]				

[ ] = total secretion in γ/day.

In the human being, PETERSON[45], by developing his ingenious tracer dilution technique, has determined average values for 24 h secretions which, according to dietary conditions, are in the range of 200–400 γ. TAIT et al.[46], having formerly reported figures only somewhat lower, give just now an average secretion rate of only 77 γ/day[48] (Table). Studies on the prolonged administration of high doses revealed that in normal subjects, as well as in patients with Addison's disease, after an initial retention of sodium and weight gain, values turn towards normal despite further administration of aldosterone (AUGUST et al.[54–58]. This escape phenomenon, known also from long term experiments with cortexone, indicates that a continuous endogenous secretion or exogenous administration of aldosterone does not necessarily lead to sodium retention and edema formation[59]. If the normal sodium excretion often observed in patients with primary aldosteronism can be explained as a consequence of the escape phenomenon, or whether it is even justified to try to provoke such an extreme reaction in cases of secondary aldosteronism by administering exogenous hormone[60] needs further investigation. It can, however, be assumed that, even in Addison's disease, the dangers from aldosterone overdosage are not great, even during prolonged administration[53, 54], although further experience of long courses of treatment under various conditions is necessary.

The speculative statements made a few years ago, ascribing a pathogenic rôle to absolute or relative overproduction of cortexone or substance S in inflammatory diseases, hypertension, or other diseases of adaptation were subsequently refuted by experimental and clinical facts. This sequence of events should at least have the one beneficial consequence for aldosterone that similar mistakes are avoided to-day and that one becomes more careful in assuming a causative relationship to be the basis of variations which happen to occur simultaneously.

4. *The Extrarenal Effects of Aldosterone.* It cannot yet be decided whether the amelioration of the symptoms of adrenal insufficiency produced by aldosterone can mainly be ascribed to the normalisation of renal function. This is certainly the case for the decrease in the plasma non-protein nitrogen, although it is not fully understood whether it is the reduced glomerular filtration rate alone, developing during adrenal in-

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sufficiency, which is responsible for the impaired kidney function or whether other mechanisms may be involved. Very little, however, is known about the influence of aldosterone and other cortical hormones on electrolyte, especially cationic exchange outside the tubular cells of the kidney. Numerous attempts have been made to demonstrate a direct action of aldosterone on various tissues, especially on functions in which an electrolyte exchange may be involved, but the results available are not yet very convincing, perhaps because of the delayed action which the hormone exerts. In erythrocytes, active cation transport is inhibited by cardiac glycosides[61], leading to a diminished potassium influx and an increase of extracellular potassium concentration. Aldosterone was said partially to antagonise this effect of strophanthin[62], but other investigators did not observe any influence on the effect of digoxin on cation transport in isolated red cells nor was the cation movement across the erythrocyte membrane affected by aldosterone itself[63,64], although here, too, the results are contradictory[65]. The significance of an increased sodium/potassium ratio in the erythrocytes of patients with secondary aldosteronism due to cardiac failure still has to be evaluated[66].

The antagonism to acetylcholine which various corticoids exert is not specific but may only be the expression of a change of the membrane properties by steroids which lead to a partial inhibition of depolarisation (Fig. 4). The promptness of this effect also indicates its non-specific nature, as well as the fact that aldosterone acts less markedly than does cortexone.

In the nephrectomised rat, aldosterone was said to increase extracellular fluid volume and extracellular sodium concentration, acting thus in the opposite sense to adrenalectomy[32]. The data available, however, are contradictory, as other investigators did not find in the nephrectomised and simultaneously adrenalectomised animal a normalisation of the increased extracellular concentration of potassium[67].

RONDELL[68] found that isolated rings of rabbit aorta under the influence of relatively high concentrations of aldosterone (10^{-5}) develop an increased sensitivity to either nor-adrenaline or angiotensin (Fig. 5). Under similar conditions but exposed to much lower concentrations of aldosterone, the isolated heart of the rat works not only longer but is also more efficient than without the addition of the hormone which, in these experiments, has an influence on heart muscle qualitatively similar to that of the cardiac glycosides[69].

A direct effect of aldosterone on cation exchange was demonstrated in the isolated bladder of the toad in which, after a latent period of 1 h, the net sodium transport across the bladder wall was, under the influence of low hormone concentrations (2×10^{-7}), more than twice that of the controls[70]. This effect could be blocked by spironolactone in 100 times higher concentrations, which indicates that the competitive antagonism between these two steroids, assumed to occur in the distal tubular cells, may exist also in other tissues.

In the brain and in the skeletal muscle of intact mice the intra-extracellular concentration gradients of sodium and of potassium are both found to be influenced by aldosterone, indicating that the hormone may act directly on cation exchange in various tissues[71]. Recently it was claimed that the distribution of other cations, such as magnesium and calcium, may be influenced in skeletal muscle and that excess aldosterone

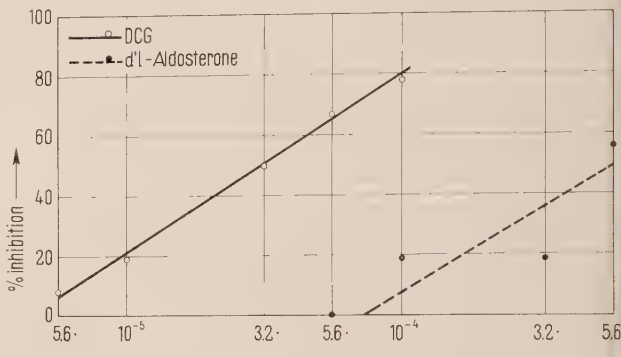

Fig. 4. Inhibition of ACh action on frog heart. Dose response curve for the inhibitory effect of cortexone-glucoside (DCG) and aldosterone on acetylcholine action in the frog heart.

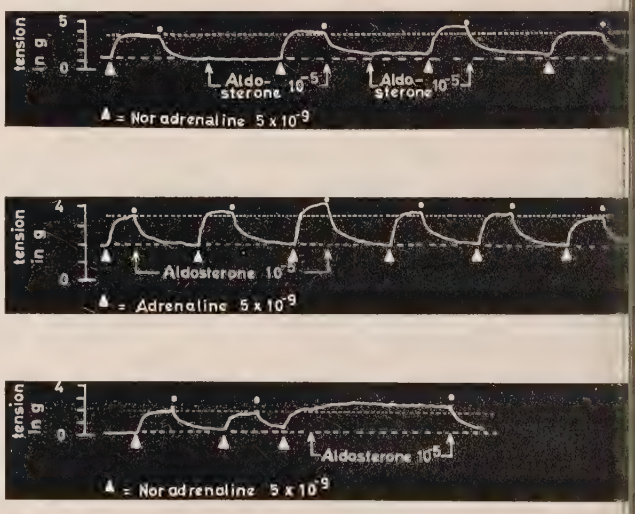

Fig. 5. Tension development in isolated rabbit aortic rings.

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eads to magnesium depletion[72,73]. Much more work, nowever, has to be done in this direction, especially vith various doses of aldosterone under different condi-ions, before assumptions regarding extrarenal activi-ies become established.

In searching for an interpretation of all the different indings, we become aware that too many gaps and ontradictions militate against this attempt. It has to e stressed that cells of different tissues may react uite differently to aldosterone under physiological onditions and differently again when isolated. Hence ll generalisations based on single observations should e avoided. The greatest obstacle is the fact that aldo-terone, like other corticoids, exerts – if any – only nodifying influences on various tissue functions and hat a deficit leads to changes which are only just within the limits of accuracy of most of the methods vailable to us. Furthermore, it cannot be expected hat an excess of an agent with a permissive or modi-ying influence, when added to an intact organism, will mprove a function beyond normal.

5. *Antitoxic Effects of Aldosterone.* Although, soon fter its isolation, aldosterone was shown to have some ortisol-like activity as regards glycogen fixation in the ver[74] and diminution of the circulating eosino-hils[75,76], these actions were only demonstrable with oses much higher than those necessary for the effects n electrolyte excretion. Furthermore, even with such igh doses of aldosterone, no cortisone-like effect on he formation of foreign body granuloma or other anti-nflammatory activity was observed[77]. It was there-ore surprising to find that under certain experimental onditions, aldosterone may exert antitoxic effects which are in the same range or even more pronounced han those of the glucocorticoids. These investigations tarted with an observation made by MEIER and BEIN[78] that, in the adrenalectomised cat, the impaired eaction to adrenaline could only be restored by the ad-ministration of aldosterone but not by cortexone or by ther corticoids. However, this is not the case in the log, whose response to nor-adrenaline is not influenced y adrenalectomy[79]. A partial protective effect of ldosterone against Brucella toxin was observed in nice[80]. Recently, BEIN and JAQUES[81] found that the hock-like stage which develops in cats, rats, or guinea igs after administration of bacterial endotoxins can e avoided or markedly improved by giving aldoste-one. In these experiments, aldosterone was about 30 imes more active than either prednisone or cortisol (Fig. 6). Not only the toxic symptoms produced by popolysaccharides, but also those elicited by other oxins, such as snake venom, or the shock induced by rypsin were reduced in intensity by aldosterone. urthermore, aldosterone restored the pressor response o adrenaline and to nor-adrenaline, which after the in-ection of polysaccharides may be reversed or markedly iminished.

In addition, it was possible to demonstrate an antitoxic effect of aldosterone against endotoxins in quite a different experimental set-up. In the embryonated egg, bacterial polysaccharides are highly toxic and lead to the death of the chick embryo in concentrations as low as 10^{-10}. By giving aldosterone simultaneously in concentrations of 10^{-6}, the toxicity of the polysaccharide is definitely reduced to the same extent as by cortisone (Fig. 7), while dexamethasone shows the same

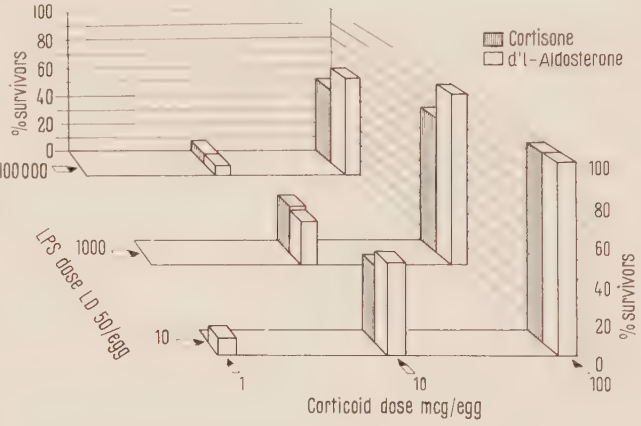

Fig. 6. Protection against bacterial endotoxins by corticoids in the cat. BEIN and JAQUES[81].

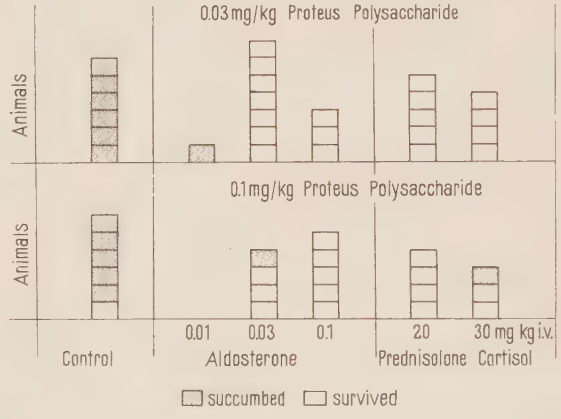

Fig. 7. Protection of corticoids against bacterial endotoxins in the embryonated egg. WYLER, KRADOLFER and GROSS[82].

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degree of protection even at concentrations 1000 times lower[82]. Some degree of antagonism against polysaccharides may also be seen in cultures of chicken leucocytes, where the enhancement of leucotaxis produced by bacterial endotoxins is reduced by aldosterone, although not to the same extent as it is by cortisol-like compounds[83]. Whether the stimulating effect of aldosterone on leucocyte agglutination and its inhibition by cortisol are of a specific nature, is however, doubtful[84].

The mechanism of action by which aldosterone may influence toxic reactions provoked by bacterial lipopolysaccharides is not elucidated so far, but it cannot be excluded that disturbances of cell permeability leading to altered distribution of sodium and potassium in the extra- and intracellular compartment of various tissues may be involved. In spite of the lack of a satisfactory explanation for this new effect of aldosterone, on the basis of the experimental data available to-day, it seems to be justified to try aldosterone in conditions of severe shock, especially if toxins are involved in the etiology and if the response to blood pressure elevating substances is reduced or abolished.

This brings us back to the introductory remark that the current characterisation of aldosterone, limiting its function to partial control of tubular cation exchange, needs reconsideration and that other aspects of its importance to the organism under physiological and pathological conditions are worth studying with greater intensity. This not only holds true for experimental but also for clinical medicine.

Zusammenfassung. Es wird eine Übersicht über die Wirkungen von Aldosteron unter physiologischen und verschiedenen experimentellen Bedingungen gegeben und gezeigt, dass das Hormon nicht nur die tubuläre Nierenfunktion, sondern auch extrarenale Vorgänge beeinflusst.

1. Aldosteron fördert die Rückresorption von Natrium in den distalen Tubuli der Niere sowie die Ausscheidung von Kalium und Wasserstoffionen, wobei eine gewisse Latenzzeit bis zum Eintreten eines signifikanten Effektes charakteristisch ist.

2. Am adrenalektomierten Tier gelingt es, durch einmalige Gabe hoher Dosen einen Zustand von schwerer Nebenniereninsuffizienz während mehrerer Tage zu unterdrücken.

3. Längerdauernde Anwendung hoher Dosen von Aldosteron führt beim Menschen oder beim Hund zu einem «escape»-Phänomen in bezug auf Natrium- und Wasserretention.

Zur Erzielung einer Blutdrucksteigerung an der Ratte sind unter gleichen Versuchsbedingungen relativ höhere Dosen von Aldosteron als von Cortexon notwendig.

4. An isolierten Geweben oder Organen (Erythrocyten, Harnblase, Froschherz, Kaninchenaorta) lassen sich erst mit relativ hohen Konzentrationen identische Effekte hervorrufen.

5. Aldosteron besitzt antitoxische Wirkungen gegenüber Polysacchariden bakteriellen Ursprungs, die mit Hilfe verschiedener Versuchsanordnungen nachweisbar sind und teilweise die für Cortisol oder Prednison bekannten ähnlichen Effekte übertreffen.

[83] B. SCHÄR, Personal communication.
[84] J. D. HARTMAN and W. F. GORDON, Amer. J. Physiol. *196*, 279 (1959).

Brèves communications – Kurze Mitteilungen – Brevi comunicazioni – Brief Reports

Les auteurs sont seuls responsables des opinions experimées dans ces communications. – Für die kurzen Mitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed by their correspondents.

6-Azacytidine - A New Antimetabolite

In the course of a programme of study of antimetabolites of the principal components of the nucleic acids[1], we have now synthesised 6-azacytidine by the following procedure: 6-Azauridine[2] was converted into the tribenzoyl derivative (I), and this, on treatment with phosphorus pentasulphide in dry pyridine, directly afforded the 4-thioderivative (II), which on ammonolysis gave 6-azacytidine (III) m.p. 215°. The same product was obtained when the de-benzoylated thiocompound (IV) was ammonolysed.

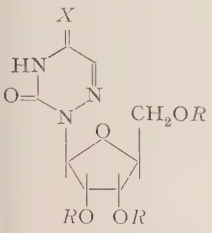
6-Azacytidine has been found to be an antimetabolite of the pyrimidine components of the nucleic acids. Its antibacterial activity against *E. coli* has been shown to be superior to that of 6-azauridine and it has also been found to inhibit the growth of some experimental tumours. The toxicity of 6-azacytidine, like that of 6-azauridine, is very low.

A more detailed account of these findings will be published in due course in Collection of Czech. Chem. Commun.

[1] F. ŠORM, A. JAKUBOVIČ, and L. ŠLECHTA, Exper. *12*, 271 (1956). – F. ŠORM and H. KEILOVÁ, Exper. *14*, 215 (1958). – J. ŠKODA and F. ŠORM, Coll. Czech. Chem. Commun. *21*, 1328 (1956). – J. ŠKODA, J. KÁRA, Z. ŠORMOVÁ, and F. ŠORM, Biochem. biophys. Acta *33*, 579 (1959). – J. GUT, J. MORÁVEK, C. PARKÁNYI, M. PRYSTAŠ, J. ŠKODA, and F. ŠORM, Coll. Czech. Chem. Commun. *24*, 3154 (1959).

[2] J. ŠKODA, V. F. HESS, and F. ŠORM, Exper. *13*, 150 (1957); Coll. Czech. Chem. Commun. *22*, 1330 (1957).

[3] Institute of Organic Chemistry, Academy of Science of the Ukrainian SSR, Kiev.



I (X = O; R = Benzoyl)
II (X = S; R = Benzoyl)
III (X = NH; R = H)
IV (X = S; R = H)

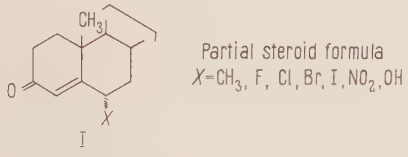
Zusammenfassung. 6-Azacytidin wurde synthetisiert; es erwies sich als Antimetabolit.

F. Šorm, J. Smrt, and V. Černeckij [3]

Department of Organic Synthesis, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague, November 3, 1960.

The Ultraviolet Light Absorption Spectra of Steroid C-6 Substituted-Δ^4-3-Ketones [1]

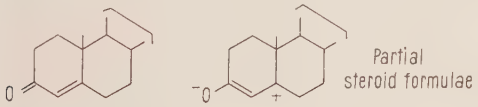
The basic structures under consideration are illustrated by formula I [2].



I

In the absence of extraneous factors (e.g. 11-keto substituents) a steroidal Δ^4-3-ketone, unsubstituted at C-2 or C-6, in alcohol solution exhibits maximum absorption in the ultraviolet at 240–242 mμ [3]. Substitution of electronegative groups at C-6 gives rise to either bathochromic or hypsochromic shifts in the absorption maximum. Before detailing the individual cases, the principal factors affecting the ultraviolet light absorption properties of C-6 substituted Δ^4-3-keto steroids will be discussed.

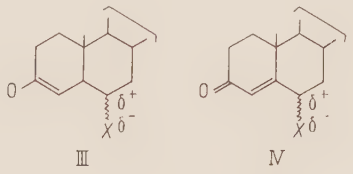
The excited state of Δ^4-3-keto steroids. The principal high intensity band of Δ^4-3-keto steroids observed in the 240 mμ region is the K band (N→V transition) and is attributed to the formation of the excited state (I→II) wherein the charged dipolar form dominates.



I II

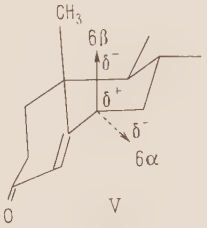
Possible factors affecting transition energy (Δ^E) of C-6 substituted Δ^4-3-keto steroids. a) *Planarity.* For effective electronic transition between the ends of a dipole it is necessary that the chromophore, in this case the unexcited unsaturated ketone, be essentially uniplanar. Steric factors which result in distortion of the chromophore plane may affect the energy requirement necessary to reach the excited state. *A priori* it might have been anticipated that a bulky 6β-axial substituent would cause a distortion of the chromophore by virtue of the 1,3-diaxial non-bonded interactions between such a group and the C-10 methyl group. Anticipating the discussion which follows it may be noted that the effect on the ultraviolet maximum of this 1,3-diaxial interaction can only be minimal.

b) *Inductive effect.* The inductive effect of an electron-withdrawing group γ-substituted to an α,β-unsaturated ketone will oppose the polarization inherent in the excited state by charge repulsion between the C-5 and C-6 electron deficient centres (cf. III). Further, an electronegative γ substituent will, by placement of a partial positive charge on the γ-carbon atom (cf. IV) oppose the mobilization of the π-electrons necessary to reach the excited state.



III IV

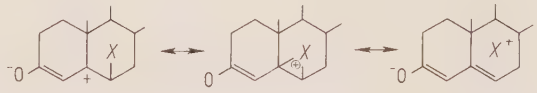
The geometry of the system is such that if the substituent at C-6 occupies the β-axial position its interaction with the dipole of the α,β-unsaturated ketone system, is of greater magnitude than that of an identical substituent in the α-equatorial position, cf. Figure V. Thus the inductive effect of a 6β substituent will be greater than that of the corresponding 6α substituent.



V

Although no such cases have been reported, one may predict that C-6 substitution of a Δ^4-3-ketone by a strong electron releasing group would result in a lowering of the excitation energy and a consequent bathochromic shift in the position of maximum absorption (K band) in the ultraviolet spectrum.

c) *Neighbouring group participation in the excited state.* If a substituent at C-6 is in such a position that it may with minimal movement interact with the unshared orbital of the excitation state C-5 carbonium ion and if the substituent is capable of accepting a positive charge, participation in the excited state is possible (Fig. VI).



VI

This will result in a lowering of the excitation energy due to an extension of the effective length of the conjugated

[1] This paper is considered to be Steroids CLXI in the series from these laboratories; part CLX, C. Casas Campillo and L. F. Bojalil, American Review of Respiratory Diseases, in press.

[2] For leading references to the preparation of compounds of this type cf. A. Bowers, E. Denot, M. B. Sanchez, L. M. Sanchez-Hidalgo, and H. J. Ringold, J. Amer. chem. Soc. *81*, 5233 (1959).

[3] For an excellent compendium and review of the ultraviolet absorption spectra of steroids, covering the literature into 1952, see L. Dorfman, Chem. Rev. *53*, 47 (1953).

system[4] (as well as resonance stabilization of the excited state).

It would appear that such participation is a major factor in the ultraviolet maximum of 6β-chloro, bromo and iodo-Δ^4-3-keto steroids.

6-Methyl-Δ^4-3-ketones. It is evident from the Table that a methyl substituent in either the 6α- or 6β-configuration exerts little or no effect on the unsaturated ketone absorption band in the 240 mμ region. This indicates that the weak electron releasing properties of this group are insufficient to exert any bathochromic spectral shift. No other factor need be considered in interpreting the spectral maximum of a 6-methyl-Δ^4-3-ketone.

6-Halo-Δ^4-3-ketones. Substitution by a 6α-halo group results in a hypsochromic shift of −4 to −5 mμ in the case of fluoro or chloro and a slightly lower shift of −3 to −4 mμ in the case of bromo. It is clear that this change is due to the inductive effect of the halogen substituents (formulae III and IV) increasing the excitation state energy requirement. The minimal difference in the effects of the three halogen substituents is paralleled by the essentially similar ionization constants of the corresponding α-halo acetic acids, acetic acid pK 4.76, α-fluoroacetic acid pK 2.66, α-chloroacetic acid 2.81, α-bromoacetic acid 2.87.

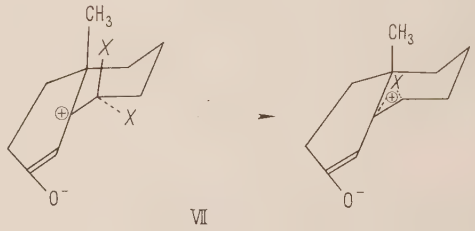
With respect to 6β-halo substituents it is apparent that in some cases more than one effect is operative. A hypsochromic effect of −5 to −8 mμ is noted for fluorine, essentially no effect is found for chlorine (0 to −1) while a bathochromic effect of +6 to +8 mμ for bromine and +11 to +13 mμ for iodine is noted. In the case of 6β-fluoro (axial) it appears that the inductive effect is the only important factor to be considered. The hypsochromic shift is greater than that of the α-fluoro substituent in agreement with the considerations noted above. By analogy with the fluoro case the inductive effect of the 6β-chloro, bromo and iodo atoms must be as great or greater than that of the corresponding 6α-substituent but this electron withdrawal effect is completely overshadowed by a second effect.

Influence of C-6 substituent on principal ultraviolet maximum of Δ^4-3-keto steroids

Substituent at C-6	$\Delta\lambda_{max}$	
	α	β
CH_3[a]	0 to +1	−1 to +1
F[b]	−4 to −5	−5 to −8
Cl[c]	−4 to −5	0 to −1
Br[d]	−3 to 4	+6 to +8
I[d]	No data	+11 to +13
OH[e]	0 to −1	−3 to −6
NO_2[f]	−7 to −9	−6 to −8

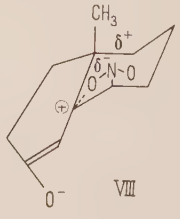
[a] H. J. RINGOLD, E. BATRES, and G. ROSENKRANZ, J. org. Chem. *22*, 99 (1957).
[b] A. BOWERS, L. C. IBÁÑEZ, and H. J. RINGOLD, Tetrahedron *7*, 138 (1959).
[c] H. J. RINGOLD, E. BATRES, A. BOWERS, J. EDWARDS, and J. ZDERIC, J. Amer. chem. Soc. *81*, 3485 (1959) and unpublished observations from these laboratories.
[d] A. BOWERS, E. DENOT, and R. BECERRA, J. Amer. chem. Soc. *82*, 4001 (1960) and unpublished observations from these laboratories.
[e] cf. [3], p. 72.
[f] A. BOWERS, M. B. SÁNCHEZ, and H. J. RINGOLD, J. Amer. chem. Soc. *81*, 5234 (1959). – A. BOWERS, L. C. IBÁÑEZ, and H. J. RINGOLD, J. Amer. chem. Soc. *81*, 3707 (1959).

The second factor operative in the 6β-halo series is the participation (see Fig. VI) of the halogen atom in the excited state which results in a bathochromic shift compared to the α-substituent. Participation appears to be negligible in the case of fluorine and then increases with decreasing electronegativity of the halogen atom (i.e. I > Br > Cl). As pointed out above, the neighbouring group must be in such proximity to the unshared orbital of the C-5 transition state carbon atom that bond overlapping can occur with a minimum of movement. This requirement is readily met by a 6β-halo substituent while considerable atomic movement through space would be required by a 6α-halo substituent for participation in a uniplanar arrangement (Fig. VII). The size of the halo atom parallels the ability of the atom to accept a positive charge and both of these factors facilitate participation of the halogen atom in the excited state. Increasing participation lowers the excitation energy and raises the ultraviolet absorption maximum of the unsaturated ketone. The bathochromic effect compared to the C-6 unsubstituted compound is at a maximum of about 12 mμ in the case of iodine. This result is consistent with the work of Winstein demonstrating that iodine is a better participating group in neighbouring group reactions than bromine or chlorine[5].



VII

6-Nitro-Δ^4-3-ketones. The powerful electron withdrawing nitro group exerts, as expected, a marked hypsochromic shift ranging from −6 to −9 mμ. However, contrary to expectation, the 6β-axial substituent is not a more potent inductive group than the 6α-nitro group and the ultraviolet shift for the 6β-group (−6 to −8 mμ) is actually slightly less than for the 6α-group (−7 to −9 mμ). A possible explanation for this discrepancy may lie in charge stabilization of the C-5 transition state carbonium ion by one of the electronegative oxygen atoms of the nitro group. The geometry of this interaction would again be more favored for the axial nitro substituent than for the equatorial one, cf. Figure VIII.



VIII

[4] cf. The bathochromic effect of the primary hydroxyl group in substituted sorbyl and cinnamyl alcohols, E. H. BRAUDE and E. S. WAIGHT, *Progress in Stereochemistry* (Ed. W. KLYNE, Butterworths Scientific Publications, London 1954), Vol. I, Chapter 4, p. 154.
[5] S. WINSTEIN, J. Amer. chem. Soc. *64*, 2791 (1942) and subsequent papers.
[6] Present address, The Worcester Foundation for Experimental Biology, Shrewsbury (Mass., U.S.A.).
[7] *Acknowledgment.* The authors wish to thank Dr. G. STORK for helpful discussions.

6-Hydroxy-Δ^4-3-ketones. A 6β-hydroxy substituent exerts a hypsochromic effect of -3 to -6 mμ while a 5α-hydroxy substituent exhibits essentially no shift (0 to -1 mμ). Only inductive effects appear to be operative since it is apparent from these results that the 6β-hydroxyl group cannot be entering into a significant degree of participation.

The difference between hydroxyl group behaviour and halogen (e.g. chlorine or bromine) with respect to neighbouring group participation in the excited state is readily explained on the basis of the smaller size of the oxygen atom as well as its greater reluctance to accept a positive charge.

Zusammenfassung. Es werden die Hauptabsorptionsbanden (N→V Übergang) für eine Serie von Δ^4-3-Steroidketonen mit C-6-Substituenten diskutiert. Dabei wird versucht, die Lage der Hauptbanden dieser Verbindungen zu erklären. Besonders wird der Einfluss des induktiven Effekts und der von benachbarten Gruppen auf den angeregten Zustand berücksichtigt.

H. J. Ringold[6] and A. Bowers[7]

The Research Laboratories, Syntex S.A., Mexico, D.F., September 5, 1960.

Determination of Pentoses

Many methods have been suggested for assaying the pentoses and particularly for ribose[1–7]. The number of procedures is an obvious indication of the difficulties of such a determination.

The method now used, with marked advantage over the other, is that of Dische and Borenfreund[8], based on a modification of the phloroglucinol reaction of Tollens[9]. Even the investigators and originators of this method themselves admit that it is less sensitive (about half) than the orcinol method suggested by Bial[1], but it has the enormous advantage of greater specificity. In consideration of the latter, we decided to see if it was possible to increase the sensitivity of the Dische-Borenfreund method.

Our aim was reached after many attempts, by suitably modifying the original method of Dische and Borenfreund. The modified method is of advantage for its increased sensitivity and consists in treating the sugar solution with concentrated HCl which doubles the intensity of the colour developed by pentose and phloroglucinol with respect to that obtained in the experimental conditions suggested by Dische and Borenfreund.

New method. Reagents and technique. The new method requires the following reagents: a) Hydrochloric acid (d = 1.19); b) Glacial acetic acid; c) 0.8% aqueous glucose solution; d) Alcoholic solution of phloroglucinol, prepared by dissolving 5 g of phloroglucinol in 100 ml of ethanol 95°.

At the moment of use, 2 ml of hydrochloric acid (d = 1.19), 110 ml of glacial acetic acid, 1 ml of 0.8% glucose solution and 5 ml of alcoholic phloroglucinol solution are mixed. The mixture forms the phloroglucinol reagent and may be stored in a refrigerator at 0°C.

After preparing the reagent, 0.5 ml of a ribose solution containing from 5–25 μg of ribose are placed in a test-tube; 0.5 ml of HCl (d = 1.19) are added and the mixture shaken. Then 4.5 ml of the phloroglucinol reagent are added and the tube immersed in a boiling water bath for exactly 5 min. At the end of this period, the tube is taken out of the water-bath and immediately cooled in ice, remaining in ice until the spectrophotometric reading, which must be performed at 552 and 510 mμ, using a blank where the pentose has been replaced by 0.5 ml of distilled water. The differences between the values ob-

Tab. I. Comparison between the optical density obtained from D-ribose with the original Dische-Borenfreund method and that as here modified

D-ribose in 5.5 ml final volume	Dische-Borenfreund method			Modified Dische-Borenfreund method		
	Optical density		Difference	Optical density		Difference
	552 mμ	510 mμ		552 mμ	510 mμ	
5 μg	0.089	0.026	0.063	0.134	0.035	0.099
10 μg	0.164	0.044	0.120	0.285	0.074	0.211
15 μg	0.238	0.064	0.174	0.435	0.116	0.319
20 μg	0.320	0.080	0.240	0.585	0.165	0.420
25 μg	—	—	—	0.705	0.190	0.515

Tab. II. Optical density (O.D.) obtained in treating 10 μg of ribose alone and in the presence of 30 μg of fructose, galactose, glucosamine, mannose and glucuronic acid, respectively, according to the phloroglucinol reaction

Quantity of sugar in 5.5 ml final volume	Dische-Borenfreund method		Difference	Modified Dische-Borenfreund method		Difference
	O.D. at 552 mμ	O.D. at 510 mμ		O.D. at 552 mμ	O.D. at 510 mμ	
Ribose 10 μg	0.145	0.035	0.110	0.280	0.065	0.215
Ribose 10 μg + fructose 30 μg	0.150	0.044	0.116	0.325	0.102	0.223
Ribose 10 μg + galactose 30 μg	0.150	0.037	0.113	0.305	0.087	0.218
Ribose 10 μg + glucosamine 30 μg	0.144	0.034	0.110	0.282	0.067	0.215
Ribose 10 μg + mannose 30 μg	0.155	0.040	0.115	0.325	0.095	0.230
Ribose 10 μg + Gluc. acid 30 μg	0.180	0.042	0.138	0.340	0.089	0.251

[1] M. Bial, Dtsch. Med. Wschr. *28*, 253 (1902).
[2] G. Embden and M. Lehnartz, Z. physiol. Chem. *201*, 149 (1931).
[3] Z. Dische and K. Schwarz, Mikrochim. Acta *2*, 13 (1937).
[4] H. K. Barrenscheen and A. Peham, Z. physiol. Chem. *272*, 81 (1942).
[5] H. G. Albaum and W. W. Umbreit, J. biol. Chem. *167*, 369 (1947).
[6] W. Mejbaum, Z. physiol. Chem. *258*, 117 (1939).
[7] A. Bonsignore, G. Conte, and M. Orunesu, G. Biochim. *1*, 383 (1951–52).
[8] Z. Dische and E. Borenfreund, Bioch. biophys. Acta *23*, 639 (1957).
[9] H. J. Wheeler and B. Tollens, Liebigs Ann. Chem. *254*, 329 (1889).

tained at 552 mμ and 510 mμ correspond to the optical density of the ribose.

In order rapidly to convert the values of the optical densities into μg of sugar, it is advisable to prepare a calibration curve.

The difference in sensitivity of the original DISCHE-BORENFREUND method and of that proposed here is quite obvious from the data reported in Table I, which clearly demonstrates the higher sensitivity (almost double) of our method over that of the above-mentioned research workers. Quite naturally, we also studied the possibility of interference of other substances, among which we examined fructose, mannose, galactose, glucuronic acid and glucosamine, substances frequently mixed with ribose in biological material. We did not consider glucose, as it is already present to a large extent in the phloroglucinol reagent where it has the function of stabilizing the colour. This test was carried out in parallel, using our modified procedure and the original DISCHE-BORENFREUND method. We first of all tested the above substances singly mixed with ribose (Tab. II). It can be seen that the substances tested interfer to a negligible or only slight extent. Only glucuronic acid interfers both in our method and in that of DISCHE-BORENFREUND.

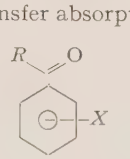
Riassunto. Si descrive una modificazione del metodo di DISCHE e BORENFREUND per la determinazione dei pentosi, che raddoppia all'incirca la sensibilità. La modifica più importante consiste nel trattamento iniziale della soluzione zuccherina con HCl concentrato e nella riduzione a soli 5 min del tempo di immersione in bagno maria bollente dopo l'aggiunta del reattivo al floroglucinolo.

L. BOLOGNANI, G. COPPI, and V. ZAMBOTTI

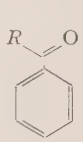
Istituto di Chimica biologica dell'Università di Pavia (Italy), October 5, 1960.

The Electron-Transfer Absorption of Substituted Benzenes

The electronic spectrum of the composite molecule containing unlike conjugated chromophores has been interpreted in terms of wave functions for the separated fragments[1,2]. These functions describe electronic transitions involving locally excited (L.E.) states and electron transfer (E.T.) states. Consideration of the principal E.T. bands of acetophenone, benzaldehyde, benzoic acid and their mono-substituted derivatives reveals a marked distinction between the *ortho-* and *meta*-disubstituted benzenes and their *para*-isomers. Thus, not only do the *para*-isomers of (I) (where X = alkyl, ring residue, –OH, OMe, $–O^-$, –Hal, $–NR_2$) absorb at longer wavelengths than the *ortho-*[3] or *meta*-isomers, but further, the spectra of the latter class are characterised by the appearance of *two* E.T. bands, whereas the *para*-compounds display a single band due to such a transition. These observations are in accord with a wave-mechanical treatment of electron-transfer absorption[4].



(I)



(II)

The fundamental nature of the these effects inclines us to the view that the principal E.T. band of the basic chromophore (I) should be affected in a predictable manner by the nature and position of X[5]. We have therefore selected parent values for the chromophore (II) listed in Table I, which, in conjunction with the increments in Table II allow of calculation of the position of the principal E.T. band of a variety of *poly*-substituted aromatic ketones, aldehydes, and carboxylic acids to within 5 mμ. The choice of solvent is most important and we have

Tab. I. Parent values for chromophore (II) [EtOH solution]

R.	λ^* [a] (mμ)
Alkyl or ring residue	246
OH or OAlk	230
H	250

[a] $\lambda^* = \lambda_{max}$ in EtOH.

Tab. II. Calculation of the principal E.T. band of the *poly*-substituted benzenes, Ar.CO*R*. (EtOH solution)

R.		λ^* [a] (mμ)
Alkyl or ring residue	*o-*, *m-*	3
	p-	10
–OH, –OAlk	*o-*, *m-*	7
	p-	25
$–O^-$	*o-*,	11
	m-	20
	p-	78
–Cl	*o-*, *m-*	0
	p-	10
–Br	*o-*, *m-*	2
	p-	15
$–NH_2$	*o-*, *m-*	15
	p-	58
–NHAc	*o-*, *m-*	20
	p-	45
$–NMe_2$	*o-*, *m-*	20
	p-	85

[a] $\lambda^* = \lambda_{max}$ in EtOH.

[1] H. C. LONGUET-HIGGINS and J. N. MURRELL, Proc. Phys. Soc. *68A*, 601 (1955).

[2] J. N. MURRELL, J. chem. Soc. *1956*, 3779.

[3] The presence of bulky *ortho*-substituent(s) may lower the *intensity* of the E.T. band to a considerable extent, or even remove the band completely from the spectrum. This effect may be accompanied by a wavelength shift (blue *or* red) and has been studied in a unified molecular orbital treatment by E. HEILBRONNER and R. GERDIL [Helv. chim. Acta *39*, 1996 (1956)]. The present treatment allows examples of severe steric repulsion to be recognised only with respect to wavelength shift, but does not appear to be unduly limited by such considerations.

[4] J. TANAKA and S. NAGAKURA, J. chem. Phys. *24*, 1274 (1956).

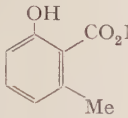
[5] Indeed, such a correlation was sought in the valuable study of substituted benzenes by DOUB and VANDENBELT[6]. However these authors used a hypothetical base line of 180 mμ as a means of deriving substitutional parameters, implying no fundamental difference between benzenoid L.E. and E.T. absorption. Further no allowance was made for the varying effect of the disposition of substituents in the *ortho-* or *para*-position with respect to a *–M* group.

[6] L. DOUB and J. M. VANDENBELT, J. Amer. chem. Soc. *69*, 2714 (1947); *71*, 2414 (1949); *77*, 4535 (1955).

selected ethanol as standard since the effects of intramolecular chelation are minimised in this medium for the particular absorption band under review[7].

We illustrate the use and scope of the method by some examples of varying complexity.

Example 1. Methyl 6-methylsalicylate (I)

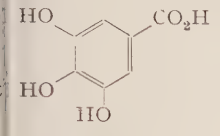


(I)

$\lambda^{*8}_{calc} = 230 + 7(ortho\text{-OH}) + 3(ortho\text{-Alk}) = 240$ mμ

$\lambda^{*}_{obs} = 244$ mμ (ε 10000)[9]

Example 2. Gallic acid (II)

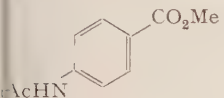


(II)

$\lambda^{*}_{calc} = 230 + 2\times7(meta\text{-OH's}) + 25(para\text{-OH}) = 269$ mμ

$\lambda^{*}_{obs} = 270$ mμ (ε 8000)[10]

Example 3. p-Acetylamino methyl benzoate (III)

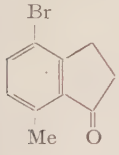


(III)

$\lambda^{*}_{calc} = 275$ mμ

$\lambda^{*}_{obs} = 274$ mμ (ε 15000)[11]

Example 4. 4-Bromo-7-methylindanone (IV)

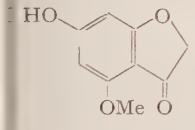


(IV)

$\lambda^{*}_{calc} = 246 + 3(ortho\text{-Me}) + 3(\text{ortho-ring residue}) + 2(meta\text{-Br}) = 254$ mμ

$\lambda^{*}_{obs} = 254$ mμ (ε 10000)[12]

Example 5. 6-Hydroxy-4-methoxycoumaranone (V)

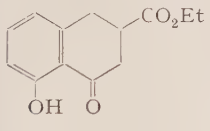


(V)

$\lambda^{*}_{calc} = 246 + 14(2\times ortho\text{-OR}) + 25(para\text{-OH}) = 285$ mμ

$\lambda^{*}_{obs} = 284$ mμ (ε 22500)[13]

Example 6. Ethyl 4-methyl-5-chloro-8-hydroxy-tetralone-3-carboxylate (VI)

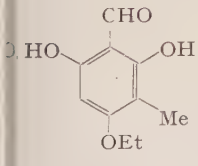


(VI)

$\lambda^{*}_{calc} = 256$ mμ

$\lambda^{*}_{obs} = 257$ mμ (ε 8000)[14]

Example 7. 2,6-Dihydroxy-3-methyl-4-ethoxybenzaldehyde (VII)



(VII)

$\lambda^{*}_{calc} = 292$ mμ

$\lambda^{*}_{obs} = 295$ mμ (ε 21950)[15]

Extension to benzonitrile by using the basic value of 224 mμ for this system reveals that the increments in Table II may be transposed to certain other series, e.g. p-aminobenzonitrile has λ^{*}_{calc} 279 mμ and absorbs at 277.5 mμ (ε 24000)[16]. Complete details of this survey will appear elsewhere.

Zusammenfassung. Eine allgemeine Methode für die Berechnung der Hauptabsorptionsbande benzolischer Carbonylverbindungen wird zur Diskussion gestellt.

A. I. Scott

Chemistry Department, The University, Glasgow (Great Britain), October 17, 1960.

[7] Correlation involving the *less intense* E.T. band of the *ortho*-disubstituted series on changing from hydrocarbon to hydroxylic solvent have already provided useful information in natural product studies [e.g. L. H. Conover, Chem. Soc. Special Publications *5*, 48 (1956). – R. A. Morton and A. L. Stubbs, J. chem. Soc. *1940*, 1347].

[8] $\lambda^{*} = \lambda_{max}$ in EtOH.

[9] C. J. W. Brooks, personal communication.

[10] J. E. Hay and L. J. Haynes, J. Chem. Soc. *1958*, 2231.

[11] P. Grammaticakis, Bull. Soc. Chim. Fr. Docum. *18*, 220 (1951).

[12] R. A. Friedel and M. Orchin, *Ultraviolet Spectra of Aromatic Compounds* (Wiley, New York 1951).

[13] L. A. Duncanson, J. P. Grove, J. MacMillan, and T. P. C. Mulholland, J. chem. Soc. *1957*, 3555.

[14] H. Muxfeldt, Chem. Ber. *92*, 3122 (1959).

[15] R. A. Morton and Z. Sawires, J. chem. Soc. *1940*, 1052.

[16] A. Burawoy and J. P. Critchley, Tetrahedron *5*, 340 (1959).

Inhibition of Tyrosinase and Uricase Activity by Ultraviolet Radiation

Recent studies from this laboratory have shown that ultraviolet (UV) radiation at 2537 Å was capable of destroying both the *in vitro* oxidase activity of serum as well as the oxidative activity of the copper protein ceruloplasmin[1,2]. The latter is the component of serum thought to be responsible for the oxidase activity. Furthermore, the logarithm of the relative enzyme activity was found to be proportional to the incident energy of the UV radiation within the range studied. It was also found that as the oxidase activity fell with increasing dose of UV radiation, the bound copper content[3,4] of the ceruloplasmin and serum samples decreased while the direct-reading copper content[4] of the samples increased. Several other copper enzymes were studied to see a) what, if any, is the effect of UV irradiation on them and b) if there was an effect on the activity, did a relationship exist between loss of enzymatic activity and 'bound' copper content of the samples. The three copper enzymes chosen for study were uricase, plant tyrosinase and mammalian tyrosinase.

Plant tyrosinase used in this study was a Worthington lyophilized preparation from mushrooms. Uricase was the Worthington powder preparation. Mammalian tyrosinase was prepared as a crude extract[5] from Harding-Passey

[1] M. H. Aprison and K. M. Hanson, Proc. Soc. exp. Biol., N.Y., *100*, 643 (1959).

[2] M. H. Aprison and K. M. Hanson, Program and Abstracts of the Biophysical Society, L 11 (1959).

[3] C. J. Gubler, M. E. Lahey, H. Ashenbrucker, G. E. Cartwright, and M. M. Wintrobe, J. biol. Chem. *196*, 209 (1952).

[4] C. J. Gubler, M. E. Lahey, G. E. Cartwright, and M. M. Wintrobe, J. clin. Invest. *32*, 405 (1953).

[5] G. H. Hogeboom and M. H. Adams, J. biol. Chem. *145*, 273 (1942).

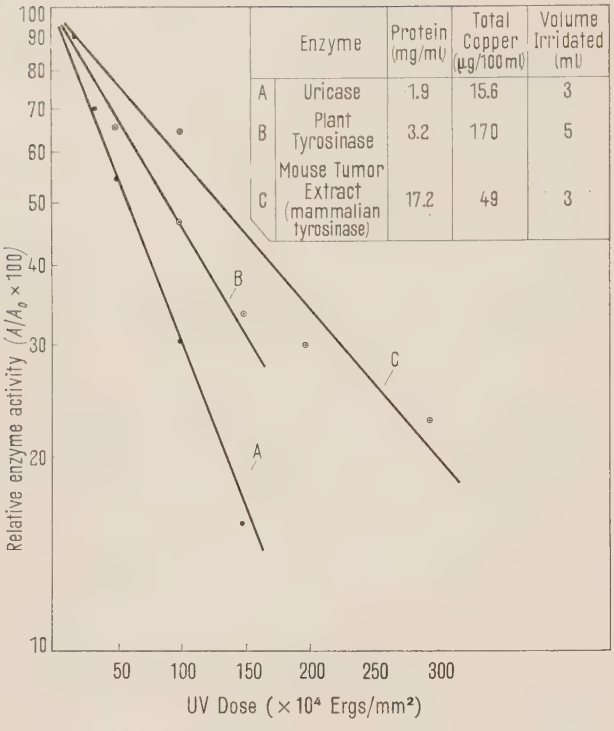

Enzyme
Protein (mg/ml)
Total Copper (µg/100 ml)
Volume Irridated (ml)
A Uricase 1.9 15.6 3
B Plant Tyrosinase 3.2 170 5
C Mouse Tumor Extract (mammalian tyrosinase) 17.2 49 3
Relative enzyme activity (A/A0 × 100)
UV Dose (× 10⁴ Ergs/mm²)

Fig. 1. Effect of UV light (2537 Å) upon the activity of uricase, plant tyrosinase and mouse melanoma extract (mammalian tyrosinase) exposed to irradiation at 0°C. Ordinate: Relative activity ($A\% = A/A_0$); abscissa: UV dose expressed as ergs/mm².

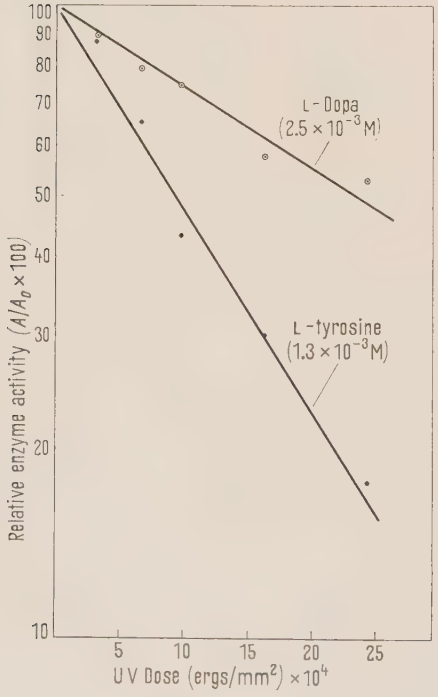


Fig. 2. Effect of UV light (2537 Å) on the activity of plant tyrosinase as assayed against L-DOPA and L-tyrosine. Optimum substrates concentrations were employed. Coordinates are the same as in Fig. 1.

mouse melanomas grown in BALB/c strain mice obtained from the Roscoe B. Jackson Memorial Laboratory.

Tyrosinase was assayed against L-tyrosine and L-dihydroxyphenylalanine (DOPA), while uric acid was the substrate for uricase. All assays were carried out at constant temperature (37°C) in a Beckman Spectrophotometer equipped with thermospacers, following the methods outlined by Worthington[6] with slight modifications. These involved running the assays at conditions of optimum substrate and pH. Tyrosinase oxidizes its substrates to an o-quinone which can be measured by an increase in optical density at 280 mμ. Uricase converts uric acid to allantoin with a resulting decrease in optical density at 290 mμ, a wavelength at which allantoin shows no absorption.

The enzyme preparations were irradiated in a Petri dish 5 cm in diameter and set in a stainless steel water bath. Ice water at 0°C and not varying more than 0.1°C was continually circulating through the water bath. After temperature equilibration was established, the sample was exposed to the UV radiation for a definite length of time. The UV source was a Mineralight ultraviolet lamp, Model V-41 (Ultraviolet Products, San Gabriel, California), and was mounted above the enzyme preparations in such a way that it could be moved in the vertical or horizontal direction. The intensity of radiation striking the solution was $(1.63 \pm 0.09) \times 10^4$ ergs/mm² as determined by the uranyloxalate actinometric method[7]. The value of the quantum yield used in the calculations was 0.60[8].

Total copper and direct-reading copper determinations were made by the method of Gubler et al.[3,4]. Bound copper content was determined as the difference between total copper and direct-reading copper.

The effect of UV irradiation on uricase, plant tyrosinase and mammalian tyrosinase is shown in Figure 1. The logarithm of the relative enzymatic activity (ordinate) is plotted against the corresponding dose of UV irradiation (abscissa). The activity of each enzyme was reduced when subjected to varying amounts of UV radiation at 2537 Å. The logarithm of the relative activity was proportional to the incident energy within the experimental error. Mathematically, the results may be represented by equation (1):

$$A = A_0 \, e^{-kD} \qquad (1)$$

where A is the enzymatic activity after a UV dose D (ergs/mm²), A_0 is the enzymatic activity of the control or nonirradiated sample, and k is a constant. The ratio A/A_0 is the relative activity.

In Figure 2, the effect of increasing doses of UV irradiation 2537 Å on plant tyrosinase activity as assayed with two different substrates is shown. The data in the upper curve were obtained using L-DOPA (2.5×10^{-3} M), while the lower curve gives results using L-tyrosine (1.3×10^{-3} M). These substrate concentrations were found to be optimal with the former being about twice the latter. The regression coefficient of the L-tyrosine curve is 0.063, approximately twice that for the L-DOPA curve which is 0.027. Thus the inhibition of the tyrosinase activity as assayed against L-DOPA is only about one half that seen when L-tyrosine is used. This may be due to the

[6] C. C. Worthington, *Worthington Enzymes*, Descriptive Manual No. 9, p. 36 (1957).

[7] F. Daniels, J. H. Mathews, and J. W. Williams, *Experimental Physical Chemistry* (McGraw-Hill, New York 1941), p. 274.

[8] W. G. Leighton and G. S. Forbes, J. Amer. chem. Soc. *52*, 3139 (1930).

[9] H. R. Mahler, G. Hubscher, and H. Baum, J. biol. Chem. *216*, 625 (1955).

fact that since both steps in the reaction, tyrosine to DOPA and DOPA to o-quinone, are under the influence of the single enzyme, tyrosinase, there is apparently double inhibition when the primary substrate, tyrosine, is used.

When the two copper methods used previously with serum and purified ceruloplasmin (50%) were employed on the samples containing the tyrosinase and uricase preparations to determine if copper to protein linkages were ruptured, clear cut results were not obtained. This is now understandable since MAHLER et al.[9] have shown that in the case of uricase, the complexing or color reagent diethyldithiocarbamate will react with copper in this system regardless of its state in the protein molecule. This is probably also true for tyrosinase. Therefore, the irradiated uricase and tyrosinase samples were dialyzed in order to see if any 'unbound' copper would pass through the membrane. As seen from the data on Table I, copper ions could pass through the membrane used in the dialysis procedure. However, the uricase activity was lost when the preparation was subjected to this treatment. Further attempts to correlate the loss of enzymatic activity with any change in copper content or state were unsuccessful.

In the case of plant tyrosinase, the dilemma could be circumvented since the enzyme was only slightly inactivated after dialysis. Therefore, after irradiation, samples subjected to different amounts of UV radiation, as well as a control, were dialyzed against copper-free distilled water at 4°C. If copper was removed or released from the bound site in the protein molecule during UV irradiation, this amount of copper would pass through the membrane and into the copper-free water on the outside. Therefore, after 24 h, the dialyzed material in the bags was removed and again assayed for oxidase activity and total copper content. These data are given in Table II and are corrected for the slight increase in volume due to dialysis. It was found that the copper concentration did not decrease proportionately as the UV dose increased. There was a difference of approximately 40 μg/100 ml of copper between the non-dialyzed sample and all the dialyzed samples. These data suggested that there must have been 40 μg of free copper/100 ml present in the original material and this was removed in each sample regardless of the amount of UV irradiation. The fall in enzymatic activity was characteristic of non-dialyzed samples subjected to similar amounts of UV irradiation.

Furthermore, it was not possible to demonstrate a change in the absorption curves (240–900 mμ) of the irradiated plant tyrosinase samples as was the case with the copper protein, ceruloplasmin. The latter had an absorption peak at 600 mμ which decreased as the oxidase activity fell and as the UV irradiation dose increased[1].

Finally, in the case of the mouse tumor extract, the difficulties encountered were such that again a direct conclusion was impossible. The optical density of this crude extract was too large to enable us to determine if slight changes in the direct-reading copper occurred after irradiation.

Tab. I. Effect of dialysis on a solution of $CuSO_4$[a] (unbound copper)

Time of dialysis (h)	Copper concentration (μg/100 ml)	% lost due to dialysis
0	200	0
5.5	14	93
24.0	3	99

[a] 5 ml samples of copper sulfate solution were used. Dialysis was done at 4°C against copper free water.

Tab. II. Effect of dialysis on copper content of UV irradiated plant tyrosinase solutions[a]

UV dose (ergs/mm²)	Dialysis time (h)	Enzymatic activity (OD/min)	Copper content (μg/100 ml)
0	0	0.0876	170
0	24	0.0825	125
48.9×10^4	24	0.0535	136
97.8×10^4	24	0.0381	122
146.7×10^4	24	0.0274	136

[a] 5 ml samples of plant tyrosinase (4 mg/ml protein) were irradiated and subsequently dialyzed against copper-free distilled water (4°C). Approximately 40 μg/100 ml of direct reading or 'unbound' copper is present; copper content of the samples does not increase with UV irradiation.

Résumé. L'activité de l'uricase et des tyrosinases d'origine végétale et animale est diminuée par irradiation UV (2537 Å). Il existe une relation exponentielle entre l'activité enzymatique relative et la quantité de rayonnement. Il n'a pas été possible de mettre en évidence une relation entre la perte d'activité de la tyrosinase végétale des échantillons irradiés et la teneur en cuivre de ces derniers.

M. H. APRISON and K. M. HANSON

The Institute of Psychiatric Research and Departments of Biochemistry and Psychiatry, Indiana University Medical Center, Indianapolis, October 24, 1960.

Specific Precipitins for Type XIV Pneumococcus Polysaccharide from *Abrus precatorius* Seeds

Several seed haemagglutinins, specific for certain human blood group characters, have been described[1]. However, many others fail to differentiate human erythrocytes[2]; the precise basis of action of these non-specific agglutinins is generally obscure. Attempts to ascribe specificity for certain 'high-incidence' human blood group antigens to some non-specific seed agglutinins were unsuccessful[3]. However, many seed agglutinins, which fail to make individual distinctions among human erythrocytes, are not altogether devoid of specificity in that they can make species distinctions among the bloods of various animals[4]. Thus various non-specific seed agglutinins can be divided into distinct classes, each class having a characteristic pattern of reactions; for example, the agglutinins of *Lens esculenta* and *Vicia faba* agglutinate rabbit and guinea pig erythrocytes but not those of several other species.

[1] G. W. G. BIRD, Brit. Med. Bull. *15*, 165 (1959).
[2] M. KRÜPE, *Blutgruppenspezifische pflanzliche Eiweisskörper (Phytagglutinine)* (Ferdinand Enke Verlag, Stuttgart 1956).
[3] G. W. G. BIRD, Vox Sanguinis *4*, 318 (1959).
[4] G. W. G. BIRD, Brit. J. exp. Path. *35*, 252 (1954).

Similarly, non-specific seed agglutinins can be classified on the basis of their capacity to be inhibited by various simple sugars[5]; thus, for example, the agglutinins of *Pisum sativum* and *Lathyrus latifolius* are inhibited by D-glucose and N-acetylglucosamine but not by D-galactose or lactose, the converse being true of the agglutinins of *Ricinus communis* and *Abrus precatorius*. There is close correspondence between the classification based on the agglutination of animal erythrocytes and that based on inhibition by sugars; for example, the agglutinins of *Ricinus communis* and *Abrus precatorius* belong to the same class when either method of distinction is adopted.

I have shown[6] that the action of the *Ricinus communis* precipitin, which is identical with the agglutinin, is apparently directed towards the basic chemical framework of the human A, B, H and Le^a blood group specific substances. This structure is so closely similar to that of the specific polysaccharide of type XIV pneumococcus that the *Ricinus* precipitin may be considered specific for this polysaccharide; this view has some experimental support[7].

Because *Abrus precatorius* and *Ricinus communis* agglutinins are classified together on the results of both animal erythrocyte agglutination and sugar inhibition reactions, it seemed likely that the activity of the *Abrus precatorius* principle is also essentially directed towards type XIV pneumococcus polysaccharide; *Abrus precatorius* seed extract was therefore studied in this light.

The extract was prepared by the method of BOYD and REGUERA[8], filtered through Whatman No. 42 filter paper and sterilised by Seitz filtration. It formed precipitates with the salivas of persons of the CEPPELLINI genotypes *SSLL*, *ssLL*, *SSll*, and *ssll*[9], and with purified A, B, H, and Le^a blood group specific substances, a purified ovarian cyst mucopolysaccharide 'Fl' devoid of ABH or Le^a activity, and purified type XIV pneumococcus polysaccharide. Titration of 1/1000 (w/v) aqueous solutions of the purified blood group specific substances, the material 'Fl' and purified pneumococcus polysaccharides of types II, III, IX, XII, XIV, and XVII against *Abrus precatorius* extract gave the results shown in the Table. The precipitation titres correspond closely with those previously obtained with *Ricinus communis* extract[6,7]; of the pneumococcus polysaccharides, only type XIV was precipitated.

OUCHTERLONY agar gel precipitation tests[10] showed that the *Abrus* precipitin, like that of *Ricinus*[6], reacts with a single substance common to the various salivas, the blood group specific substances, the material 'Fl' and type XIV pneumococcus polysaccharide. Agar gel tests also showed that *Abrus* extract, *Ricinus* extract and horse antiserum to type XIV pneumococcus give a 'reaction of identity'[11] when allowed to diffuse either against type XIV polysaccharide or the chemically similar material 'Fl', which is believed to represent the unmodified basic substrate of the human blood group specific substances[12]. Thus the activity of the *Abrus precatorius* precipitin, like that of *Ricinus communis*, is essentially directed towards the chemical configuration of type XIV pneumococcus polysaccharide.

It is noteworthy that extracts of other seeds, such as those of various species of *Datura*, which also contain powerful non-specific agglutinins for human erythrocytes but are placed in a different class to that of *Ricinus communis* and *Abrus precatorius* on the basis of animal erythrocyte agglutination and sugar inhibition characteristics, fail to precipitate any of the polysaccharides used in this investigation; the basis of action of these extracts remains to be determined.

Precipitation reactions of *Abrus precatorius* seed extract

Polysaccharide	Dilution of extract			Controls
	1/1000	1/10000	1/100000	
A-substance	2		—	
B-substance	1		—	
H-substance	3	1	—	
Le^a-substance	3	1	—	
'Fl'	3	1	tr	—
Pn II	—	—	—	—
Pn VII	—	—	—	—
Pn IX	—	—	—	—
Pn XII	—	—	—	—
Pn XIV	3	1	tr	—
Pn XVII	—	—	—	—

3:2:1:tr = degrees of precipitation; controls = extract and saline; polysaccharide and saline.

Zusammenfassung. Präzipitine aus *Abrus precatorius*-Samen scheinen folgende Spezifität zu besitzen: 1. gegen Pneumococcus-Polysaccharide des Typus XIV; 2. gegen die menschlichen blutgruppenspezifischen Substanzen A, B, H und Le^a.

G. W. G. BIRD

Armed Forces Medical College, Poona (India), August 21, 1960.

[5] A. ENSGRABER, Ber. Dtsch. Bot. Ges. *71*, 349 (1958).
[6] G. W. G. BIRD, Vox Sanguinis *4*, 313 (1959).
[7] G. W. G. BIRD, Nature, in press (1960).
[8] W. C. BOYD and R. M. REGUERA, J. Immunol. *62*, 333 (1949).
[9] R. CEPPELLINI, Proc. IVth Int. Congr. Blood Transfusion, p. 207 (1955).
[10] O. OUCHTERLONY, Proc. VIth Int. Congr. Microbiol., p. 276 (1953).
[11] The 'reaction of identity' obtained in this investigation requires elaboration; this will form the subject of a separate communication.
[12] W. M. WATKINS and W. T. J. MORGAN, Vox Sanguinis *4*, 97 (1959).

The Anthocyanins of Roses. Occurrence of Peonin

Although the garden rose contains a great range of colour varieties, only two anthocyanins have so far been identified in the petals of cyanic forms. Cyanin (cyanidin 3:5-diglucoside) was isolated from *Rosa gallica* by WILLSTÄTTER and NOLAN in 1915[1] and a pelargonidin 3:5-dimonoside (presumably the 3:5-diglucoside, pelargonin) was reported in the scarlet polyantha varieties 'Gloria Mundi', 'Prince of Orange' and 'Paul Crampel'[2,3]. The related flavonols, quercetin and kampferol were also known to occur in glycosidic form in rose petals. A third flavonol, myricetin, was recently described as occurring in about 20 Hybrid Tea varieties by SESHADRI et al.[4]. Since current work in this laboratory has shown that delphinidin and its methylated derivatives occur in association with myricetin in purple or mauve petals of a number of garden

[1] R. WILLSTÄTTER and T. J. NOLAN, Liebigs Ann. *408*, 1 (1915).
[2] G. M. ROBINSON and R. ROBINSON, Biochem. J. *28*, 1712 (1934).
[3] R. SCOTT-MONCRIEFF, J. Genet. *32*, 117 (1936).
[4] S. R. GUPTA, K. S. PANKAJAMINI, and T. R. SESHADRI, J. sci. Indian Res. B. *16*, 154 (1957).

flowers[5,6], a search for a delphinidin derivative among the anthocyanins of roses was undertaken. In particular, blooms of well established purple and mauve varieties as well as those of the latest and bluest breeding lines were examined.

No delphinidin was found and a re-examination of the rose varieties reported to contain some myricetin[4] showed that only kampferol and quercetin were present. In the course of this survey, however, a third major anthocyanin was discovered in *Rosa rugosa* and derived varieties, e.g. 'Roseraie de L'Hay'. The pinkish red petals of these plants contain cyanin and the new pigment, which was readily identified as peonin (Tab. I). Since peonin is rare and has only previously been found in quantity in peony blooms[7], its presence in pink roses provides a valuable alternative source. Other new sources are the pink flowered garden geranium (Tab. I), a plant already known to contain pelargonin and malvin[2], and dark red varieties of *Lathyrus odoratus*[8].

Of the two previously known anthocyanins of roses cyanin is the most widely distributed, being present in all but two of the hundred or so varieties examined. The pelargonidin derivative, whose identity with pelargonin has now been confirmed, occurs in a number of scarlet varieties (e.g. 'Radar' and 'Will Scarlet') besides those already mentioned. Colour in the rose is therefore mainly due to pelargonin, cyanin or peonin or to mixtures of these pigments. Traces of the related 3-glucosides accompany these 3:5-diglucosides in some varieties. Purple or mauve colours are produced by co-pigmentation of cyanin; a fact which has been established by spectral measurements of aqueous acid extracts of the appropriate varieties (Tab. II).

Zusammenfassung. Im Gegensatz zu einer früheren Mitteilung konnten wir in den Blumenblättern der Hybriden-Tee-Rose kein Myricetin finden. Delphinidin kam in keiner der geprüften Rosenblüten vor. In roten Varianten erhält man violette Farben bei der «Copigmentation» von Cyanin. Pelargonin ist in gelbroten Rosen vorhanden und Peonin wurde erstmals in der rosa Variante gefunden.

Tab. II. Co-pigmentation in mauve roses

Variety	Petal colour	Visual max in 1% aqu. HCl (in mμ)	Anthocyanidin formed on acid hydrolysis
'Belle Poitevine'	red	507	cyanidin
'Reine des Violettes'	violet	509	cyanidin
McGredy 56/944	violet blue	510	cyanidin
McGredy 55/1965	mauve	512	cyanidin

J. B. Harborne

John Innes Institute, Bayfordbury (Hertford, Herts), October 20, 1960.

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Tab. I. Identification of peonin and pelargonin

Pigment and source		Spectral max in MeOH/HCl (in mμ)		Spectral ratio 440 mμ/max (as %)	Rf values in BAW	BuHCl	1% HCl	Hydrolysis Products[a]
Peonin	authentic	274	523	13	0.31	0.10	0.17	peonidin, the 3- and 5-glucoside and glucose
	from *Rosa*	273	524	14	0.31	0.10	0.17	
	from *Pelargonium*	274	523	13	0.31	0.10	0.17	
Pelargonin	authentic	269	505	21	0.31	0.14	0.22	pelargonidin, the 3- and 5-glucoside and glucose
	from *Rosa*	266	507	20	0.31	0.14	0.23	

[a] For details of their identification see [6].

Rate of Respiration in Relation to Autogamy in *Paramecium aurelia*

It is well known that cultures of paramecia in which autogamy (self-fertilisation) and conjugation (cross-fertilisation) are prevented by daily re-isolation into fresh medium undergo a progressive process of aging. Rejuvenescence can be obtained by allowing autogamy (or conjugation) to take place. A point is finally reached, however, when rejuvenescence can no longer be obtained by this procedure and the clone is doomed to extinction[1]. The theoretical basis of this phenomenon is as yet obscure—rejuvenescence is dependent on a reproductive process involving reconstitution of the nuclear apparatus of the organism by a meiotic process (autogamy or conjugation) in which there is no segregation of genetic material since homozygous lines can be used with equal facility.

In the present investigation measurements have been made of the rate of respiration of single paramecia from serial isolation cultures before and after the occurence of autogamy in order to elucidate the nature of the metabolic changes occuring as a consequence of autogamy.

The stocks of paramecia used were selected at random and belong to two syngens (varieties) of the species *Paramecium aurelia*; namely stock 60, syngen 1 (collected originally in Burlington, Virginia), and stock 39, syngen 9 (isolated originally from the river Eure, France).

Respiration was measured by Cartesian diver microrespirometry and the apparatus and methods employed were similar to those developed by Linderstrøm-Lang[2,3] and Holter[4] with only minor modification. In order to facilitate comparison two divers only were used throughout. A single paramecium of known physiological status was loaded into the diver under sterile conditions. The charge containing the paramecium consisted of standard culture medium (an infusion of dried lettuce innoculated with a strain of *Aerobacter aerogenes*) passed through a

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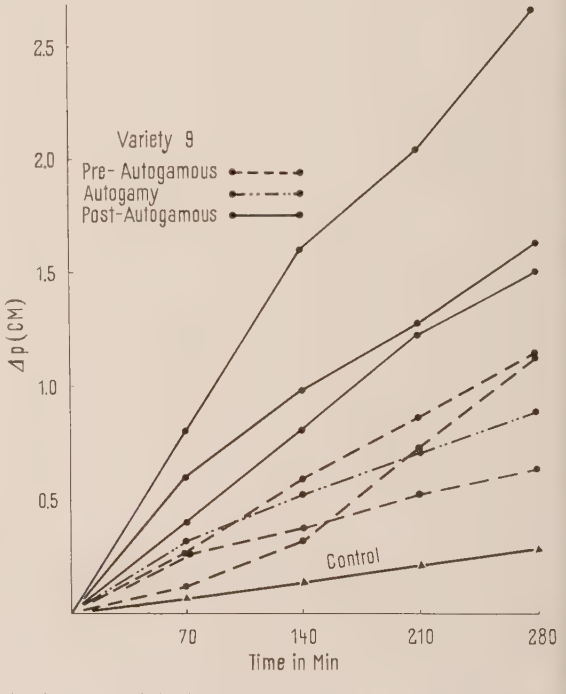
Variety	Stock	Stage	Duration of Experiment (min)	Diver	Mean Δp/h less control value (cm)	Mean oxygen consumption (μl/h)	
1	60	Pre-autogamous	280	D_1	0.098	1.531	1.227
1	60	Pre-autogamous	280	D_1	0.059	0.923	
1	60	Post-autogamous	280	D_1	0.265	4.150	4.100
1	60	Post-autogamous	280	D_1	0.259	4.050	
9	39	Pre-autogamous	280	D_2	0.168	2.535	
9	39	Pre-autogamous	280	D_2	0.173	2.599	
9	39	Pre-autogamous	280	D_2	0.063	0.955	1.823
9	39	Pre-autogamous	280	D_1	0.104	1.623	
9	39	Pre-autogamous	280	D_1	0.093	1.460	
9	39	During autogamy	280	D_2	0.117	1.762	1.544
9	39	During autogamy	280	D_1	0.085	1.326	
9	39	Post-autogamous	280	D_2	0.273	4.008	
9	39	Post-autogamous	280	D_2	0.497	7.480	
9	39	Post-autogamous	280	D_2	0.250	3.753	5.506
9	39	Post-autogamous	280	D_1	0.183	2.879	
9	39	Post-autogamous	280	D_1	0.248	3.880	
Mean Δp/h for control divers (i.e. no paramecium in diver)			480	$D_1 + D_2$	0.072		

Hemmings filter to remove the bacteria. The paramecium was freed of bacteria by repeated washings in sterile culture fluid. The respiration measurements were carried out at 26°C, which was also the temperature of incubation of the parent cultures. The pre-autogamous animals were taken from a homozygous clone which had undergone 50–150 divisions in the absence of autogamy. The post-autogamous animals were derived from the same cultures and the respiration measurements were carried out 2–3 divisions after autogamy. The procedure for controlling the occurence of autogamy in laboratory cultures has been described by SONNEBORN[5].

The Figure illustrates the results obtained with one of the divers. The respiration measurements with each diver must be plotted separately since the gradients of the lines (Δp) is a function of the diver constant. The calculated values for the rates of oxygen consumption (in μl/h) are given in the Table. These results demonstrate the feasibility of measuring the respiratory rate of single paramecia at specific phases of their life cycle. So far they indicate that there is a marked increase in oxygen consumption after autogamy in comparison with the pre-autogamous rate. The difference is statistically significant in the case of the syngen 9 figures (t test; $p < 0.001$). There is no indication in the figures at present available of an inter-stock difference of comparable magnitude. The rate during autogamy (at anlagen formation) appears to be similar to the pre-autogamous rate.

The values for the rates of oxygen consumption are of the same order as those obtained by CUNNINGHAM and KIRK[6] for *P. caudatum*, but somewhat larger than those obtained by some other workers[7–10]. It has been shown, however, that culture conditions (density) markedly affect oxygen consumption[11]. Consequently higher values would be expected in this case since the measurements were made on single animals and not on mass cultures.

In the case of plant and animal tissues it has been shown that a high respiration rate is associated with youth and that there is a progressive decline in the rate of oxygen consumption of tissues with age[12–16]. It would appear, therefore, that the aging phenomenon observed in paramecia cultures is analogous to the aging of plant and animal tissues.



Respiration rates of single paramecia of Variety 9 before, during and after autogamy (Diver D_2).

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Zusammenfassung. Die Atmung einzelner Pantoffeltierchen (*Paramecium aurelia*) aus Serien-isolierten Kulturen wurde mit Hilfe eines Cartesischen Tauchers gemessen. Die Messungen an Individuen vor und nach Autogamie (Selbstbefruchtung) zeigten, dass die Atmung nach Autogamie in signifikanter Weise ansteigt.

Da die Autogamie für das Überleben der Kulturen eine grosse Wichtigkeit besitzt, ergeben sich Parallelen zwischen den Stoffwechselveränderungen bei *Paramecium* und der Alterung der somatischen Zellen von Metazoen.

C. R. Pringle and J. M. Stewart

Zoology Department, University of Aberdeen (Scotland), November 5, 1960.

Vitamin Requirements of *Nemalion multifidum*

In axenic culture, the red alga *Goniotrichum elegans* was found to be a vitamin heterotroph[1,2]. Like many other marine organisms, it required cyanocobalamin for growth. In November 1959, success was achieved in growing another red alga in axenic culture, *Nemalion multifidum* (Weber and Mohr) J. Ag. a member of *Florideae*. The method for sterilization will be described in a later paper. The method for cultivation was the same as that earlier used by the author[2]. The alga was grown in the artificial medium ASP 6[3] with nitrate replaced by asparagine. During the first months, the alga seemed to be autotrophic. Cultures in a vitamin-containing substrate grew as rapidly as those in a vitamin-free solution. Subsequently, however, the vitamin-starved cultures became pale in colour, and additions of the vitamin solution of ASP 6, a mixture of 15 different vitamins, increased growth up to 100%. Eleven of the vitamins were sterilized together in an autoclave ('autoclaved vitamins') but the remainder, pyridoxamine, lactoflavin, folic acid and cyanocobalamin, were passed through a sterile glass filter before being added to the sterile flasks. The active substance could be located as being among these four vitamins (Tab. I). Cyanocobalamin (= B_{12}) had the strongest effect, but pyridoxamine also caused an increase in growth.

In order to determine which part of the cyanocobalamin molecule was necessary, different B_{12}-analogues were added (Tab. II). At that time, the inocula had been starved for four months and even additions of cyanocobalamin increased growth very little. However, Factor Ib (= Factor B ribosephosphate) was the more active one; while Factor B, which contains only the pseudophorphyrine ring, had no effect. *Nemalion* was very little affected by such analogues as Factor III or Factor Z1, in contrast to the behaviour of *Goniotrichum elegans*.

In the early experiments, not only pyridoxamine but also pyridoxine increased the growth of Nemalion. When added together with B_{12} or Factor Ib, pyridoxamine gave a higher yield than these compounds alone. When the inocula became more starved this effect did not appear (Tab. III).

In all these experiments, some growth occurred also in the controls; and thus a very weak vitamin production is indicated. An addition of one vitamin can thus enhance growth while there is some production of other necessary compounds but not sufficient for optimal growth. The cyanocobalamin molecule seems to be broken down before its incorporation in an enzyme and the Factor Ib molecule without the benzimidazole ring is more conveniently utilized for this purpose. The role which the B_6 vitamins play in this connection cannot yet be explained, but a cooperation between B_{12} and B_6 is known from the methionine synthesis of a B_6-less mutant of *Escherichia coli*[4].

Tab. I. Growth of *Nemalion multifidum* with different vitamins

Substance added	Dry weight in mg of algae from 6 flasks	
	Experiment 1 Incubation time 24 days	Experiment 2 Incubation time 20 days
0	6.4	5.8
All vitamins	10.6	
'Autoclaved vitamins'	8.6	
Pyridoxamine, lactoflavin, folic acid, B_{12}	13.8	9.7
Pyridoxamine	12.8	8.9
Lactoflavin	5.8	—
Folic acid	7.4	7.2
B_{12}	14.2	9.9
B_{12} + pyridoxamine	—	10.8

Tab. II. Growth of *Nemalion multifidum* with different B_{12}-analogues

Additions of B_{12}-analogues 1 µg/l	Dry weight in mg of algae from 6 flasks
0	12.7
B_{12}	14.0
Factor B	13.0
Factor III	14.0
Pseudovitamin B_{12}	13.8
Factor Ib	32.9
Factor Z1	14.3
Factor Z2	14.4
Incubation time 42 days.	

Tab. III. Growth of *Nemalion* with additions of B_{12} or Factor Ib together with pyridoxamine and B_6

Addition	Dry weight in mg of algae from 6 flasks		
	Exper. 1	Exper. 2	Exper. 3
0	4.2	4.8	2.6
B_{12}	4.8	—	—
Factor Ib	17.0	11.1	9.0
B_6	4.0	—	—
Pyridoxamine	4.2	5.6	—
$B_{12} + B_6$	5.0	—	—
B_{12} + pyridoxamine	5.8	—	—
Factor Ib + B_6	21.4	—	—
Factor Ib + pyridoxamine	23.2	10.4	7.4
Incubation time 30 days.			

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Zusammenfassung. Es ist gelungen, *Nemalion multifidum*, eine marine Floridee, in axenische Kultur zu bekommen. Die Alge wurde in einer synthetischen Nährlösung gezüchtet. Wenn sie ohne Vitamine 2 Monate kultiviert wurde, konnte ein Zusatz einer Vitaminmischung den Zuwachs verdoppeln. Die wirksamen Vitamine waren am Anfang B_{12} oder B_6; wenn die Alge aber längere Zeit in Hungerkultur gezüchtet worden war, konnte nur die B_{12}-Analoge, Factor I b, den Zuwachs erhöhen.

Lisbeth Fries

Institute of Physiological Botany, University of Uppsala (Sweden), November 21, 1960.

6-Hydroxylation: An Important Metabolic Route for α-Methyltryptamine

D,L-α-methyl tryptamine (αMT) has been found to produce LSD_{25} like effect in normal volunteers[1]. We have found that the effect of 20 mg αMT is comparable in intensity to the psychotomimetic effect of about 60 mg N,N-diethyltryptamine (DET) in man with the difference that the perceptual distortions are more and the sympathomimetic vegetative changes are less pronounced than those produced by DET[2]. Since DET has been found to produce the behavioral changes through the 6-hydroxylated metabolite[3], the question arises whether or not αMT also forms 6-hydroxylated metabolites.

Although the biochemical effects of αMT on serotonin metabolism has been extensively studied[4–6], very little is known about the metabolism of the compound itself. Since the side chain of αMT is identical with the side chain of amphetamine ($-CH_2 \cdot \underset{\displaystyle CH_3}{\underset{|}{CH}} \cdot NH_2$), the compound was expected to undergo the same microsomal deamination as amphetamine[7].

Incubating αMT with rat liver microsomes, soluble supernatant fraction, TPN and Mg^{++}[7] at 37°C resulted in the formation of 3-indolylacetone as evidenced by the paper chromatographic identification of the metabolite in the neutral ether extract with synthetically prepared 3-indolylacetone[8]. Both the metabolite and the synthetic compound gave the same Rf value in three different solvent systems and produced the same purple color reaction with Ehrlich's *p*-dimethylaminobenzaldehyde. The chromatograms of the neutral ether extract also showed another new compound which gave a fast developing blue reaction with Ehrlich's reagent and instant red color with diazotized sulfanilic acid in HCl specific for 6-hydroxy indole derivatives[9]. The Rf values of this new compound (0.83 in *n*-butanol-acetic acid and 0.90 in isopropanol-ammonia) and the color reactions were identical with those of the single metabolite obtained from synthetic 3-indolylacetone in the same microsomal enzyme system. Therefore, this compound is most probably 6-hydroxy-3- indolylacetone.

The pH of the enzymatic reaction mixture (previously extracted with ether) was then adjusted to 9.5 and extracted with *n*-butanol. When the butanol extract was shaken with dilute acetic acid, the aqueous layer contained, besides unchanged αMT[10], a new apparently basic indole derivative (Rf 0.48 in butanol-acetic acid, 0.66 in butanol-ammonia) which gave the color reactions characteristic for 6-hydroxy indoles. It is most likely that this compound is 6-hydroxy-α-methyltryptamine (6-HO-αMT).

Additional evidence for the identity of 6-HO-αMT was obtained by an experiment in which the isolated compound was again incubated with the microsomal enzyme system and yielded 6-hydroxy-3-indolylacetone by deamination.

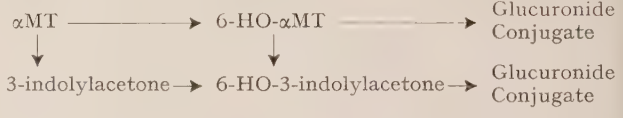
Thus the three major enzymatic products were tentatively identified as 3-indolylacetone, 6-HO-αMT and 6-hydroxy-3-indolylacetone.

The same metabolites have been found to be formed in intact animals and excreted in the urine mainly as glucuronide conjugates.

Male albino rats were injected with αMT (5 mg/kg) intraperitoneally and the urines were collected for 48 h in metabolic cages. Aliquots of the urine samples were incubated with bacterial β-glucuronidase (Sigma) in order to hydrolyze the glucuronide conjugates.

The urine samples were chromatographed without previous extraction. The chromatograms of the samples not treated with β-glucuronidase showed the presence of some unchanged αMT, a spot corresponding to 3-indolylacetone, a small spot corresponding to 6-HO-αMT and a large presumably double spot with low Rf values (0.04 to 0.20 in butanol-acetic acid and 0.20–0.45 in isopropanol-NH_3) and with typical 6-hydroxy-indole color reactions. On the chromatograms of the glucuronidase treated urine samples, this large double spot with low Rf values was absent but the spot corresponding to 6-HO-αMT was tremendously increased and another compound appeared which proved to be identical with the enzymatically prepared 6-hydroxy-3-indolylacetone.

From the evidence described above the following tentative scheme for the metabolism of αMT may be drawn:



Since 6-hydroxylated indole derivatives are more potent pharmacologically than the non-hydroxylated parent compounds[11], it is possible that one or both of the 6-hydroxy metabolites play a part in the behavioral effect of αMT.

Zusammenfassung. Der Stoffwechsel der psychotropischen Verbindung α-Methyltryptamin wurde untersucht. Die drei hauptsächlichsten Metabolite wurden *in vitro* und *in vivo* als 6-Hydroxy-α-methyltryptamin, 3-Indolylaceton und 6-Hydroxy-3-indolylaceton identifiziert.

S. Szara

Clinical Neuropharmacology Research Center, Washington (D.C.), October 4, 1960.

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In vitro and *in vivo* Activity on Catalase of Electrophoretically Pure Human Serum Albumin

It has been demonstrated[1] that protein fractions, isolated from tumoral and non-tumoral ascitic fluids and consisting for the most part of albumin, decrease the liver catalase activity in mice. Therefore we tested whether normal human serum albumin, electrophoretically pure, would show a corresponding inhibiting action.

Human albumin Behringwerke (Marburg-Lahn) 'reinst' Op. Nr. 1186 II was used. The material was dissolved in physiological saline and 0.3 ml were injected subcutaneously, at concentrations varying from 5 to 100 mg/ml. Male albino mice of the inbred strain IVB (originally from Institut Curie, Paris) weighing 20–24 g, were used. Ten mice were injected for each dose and at the same time five control animals of the same strain, sex and weight were sacrificed. The % inhibition was calculated on the basis of the values found for the controls.

The catalase activity was determined, as previously described[2], 24 h after the injection by the method of von Euler and Josephson[3], using a liver homogenate with a concentration of 1 mg of fresh liver/ml. The *in vitro* activity on crystalline catalase was also tested. Beef crystalline catalase possessing a Kf of 30000, prepared according to a method already described[4], was dissolved as a concentrated solution in 1/15 *M* phosphate buffer at pH 7.4, and then diluted to a Ko of about 1000×10^{-4} with phosphate buffer 1/150 *M* at pH 6.8.

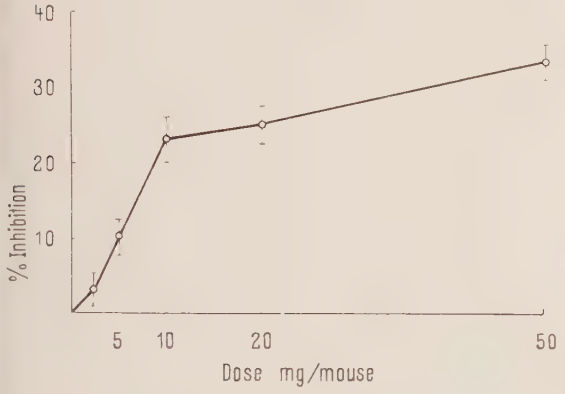

Fig. 1. *In vivo* inhibition of liver catalase by human albumin. Normal, undenatured, electrophoretically pure human albumin was injected at various dose levels to groups of 10 mice and the liver catalase activity determined 24 h after treatment. Brackets indicate the standard error.

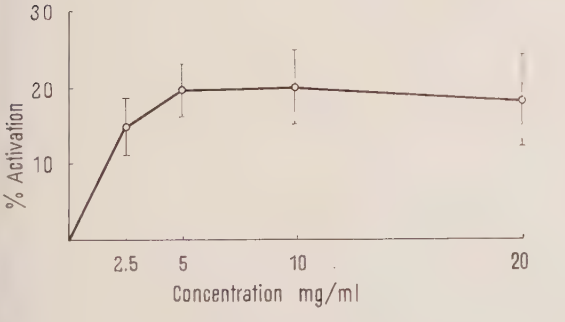

Fig. 2. *In vitro* enhancement of catalase activity by human albumin. Brackets indicate the standard error.

For the test, to 1 ml of catalase, 1 ml of albumin solution at concentrations varying from 2.5 to 40 mg/ml was added and the mixture incubated at + 2°C in a refrigerator for 1 h. After this time, the catalase activity was determined according to the method of von Euler and Josephson[3]. Owing to the instability of catalase in dilute solutions, several controls stored under the same conditions were interposed between the single tests. No relevant variations were observed among the different controls at various times.

As shown in Figure 1, there is a decrease of liver catalase activity *in vivo*, which is proportional to the amount of albumin injected. The inhibition is already present; although not significant at 5 mg dosis, it is highly significant with 10 mg of albumin. The effect is comparable to that of potent 'toxohormone' preparations.

In vitro (Fig. 2) no inhibition of crystalline catalase was observed; indeed, on the contrary, a moderate but constant activation of the enzyme activity was always noticed. The significance of this behaviour, which was shown also by some 'toxohormone' preparations, is obscure at present. The most important fact is that a pure human albumin preparation shows towards liver catalase a behaviour indistinguishable from that of a tumor extract like 'toxohormone'.

Riassunto. Albumine umane purissime dimostrano *in vivo* sulla catalasi epatica dei topi, una inibizione molto significativa fino alla dose di 10 mg/topo; *in vitro*, sulla catalasi cristallizzata, determinano un certo aumento dell'attività di difficile interpretazione.

G. Ceriotti[5]

Centro Studio Tumori di Busto Arsizio (Italy), September 16, 1960.

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[5] *Acknowledgment.* The present research was partially supported by a grant from Farmitalia S.p.A.

Insensitivity of Erythrocyte Catalase to Substances that Depress Liver Catalase

Tumors are known to depress liver catalase activity without affecting erythrocyte catalase[1,2]. According to Theorell et al.[3], this differential action is due to the different site of synthesis for the two catalases, namely the liver and the bone marrow. The tumor extract 'toxohormone' has been said to behave in a similar manner, and therefore it has been thought to represent or to contain the active principle of tumors[4,5]. As other substances are able to depress liver catalase *in vivo*, with or without a corresponding activity *in vitro*, we decided to investigate the action of some of these substances also on erythrocyte catalase.

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Comparison between liver and blood catalase inhibition, in tumor-bearing and in tyrosine and denatured albumin injected mice, 24 h after treatment

Number of mice	Treatment	% inhibition of hepatic catalase activity	Optical density of hemolysates at 575 mμ E	Catalase activity of hemolysates Ko 0.10^4	Ko/E
20	none	—	0.487 ± 0.024	507 ± 27	0.104
10	S. 180 of 10 days	30	0.465 ± 0.011	475 ± 47	0.103
16	S. 180 of 17 days	49	0.512 ± 0.004	550 ± 42	0.107
10	Tyrosine 5 mg	32	0.520 ± 0.015	517 ± 63	0.099
15	Tyrosine 10 mg	40	0.502 ± 0.006	555 ± 57	0.111
10	Denatured albumin 25 mg	37	0.570 ± 0.010	615 ± 31	0.120

The substances tested were: tyrosine which is active both *in vitro*[6] and *in vivo*[7] and a denatured albumin fraction from human neoplastic ascitic fluid, which is active only *in vivo*[8]. The latter material, before denaturation, is active also *in vitro*, but it loses this property when denatured by treatment with acetic acid at pH 4 for 1 h and is subsequently precipitated with acetone.

Tyrosine was injected subcutaneously in 10 mg doses, in 1 ml, as a fine suspension, obtained by neutralizing its acid solution.

The denatured albumin was injected subcutaneously in doses of 25 mg, suspended in 1 ml of physiological saline.

Male albino mice of the strain IVB (Institut Curie, Paris) of 18–22 g of weight were used. They were killed 24 h after the injection by decapitation, and the blood collected in citrated physiological saline. The erythrocytes were washed twice with the same solution and twice with plain saline. They were then lysed with 4 ml of distilled water, by repeated freezing and thawing. After centrifugation, the solutions were diluted to an optical density of approximately 0.500, when read on a Beckman DU spectrophotometer at 575 mμ. 1 ml of this solution was poured into 50 ml of 0.01 N H_2O_2 and catalase activity determined by the method of VON EULER and JOSEPHSON[9]. The catalase activity was referred to the hemoglobin content, by the ratio Ko to the optical density at 575 mμ. The activity of liver catalase was also determined using 1 ml of a homogenate diluted with phosphate buffer 1/150 M, pH 6.8, to have 1 mg of fresh liver/ml.

As control, normal healthy mice, and mice bearing Sarcoma 180, 10 and 17 days after implantation, were used.

As appears from the Table, the erythrocyte catalase is not affected either by tyrosine or by denatured albumin; on the contrary, there is a marked decrease of liver catalase activity for both treatments. The picture is therefore superimposable to that given by tumors.

Apparently, 'toxohormone' is not the only material acting like tumors, and it is not sufficient to observe a depression of liver catalase, without a corresponding decrease of erythrocyte catalase, to correlate the activity found with that of living tumors.

Riassunto. La tirosina che inibisce *in vivo* l'attività della catalasi epatica dei topi ed *in vitro* l'attività della catalasi cristallizzata, ed una frazione di albumine denaturate attiva sulla catalasi solo *in vivo*, si sono dimostrate inattive sulla catalasi eritrocitaria.

A. AGRADI[10]

Centro Studio Tumori di Busto Arsizio (Italy), September 21, 1960.

[6] G. CERIOTTI, L. SPANDRIO, and E. BERTI, Biochim. biophys. Acta *23*, 362 (1957).
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[10] *Acknowledgment.* The present research was partially supported by a grant from Farmitalia S.p.A.

Étude électrophorétique concernant les protéines et les acides nucléiques tissulaires a propos de l'inhibition de la cancérogenèse

L'inhibition de la cancérogenèse par administration d'implants cancéreux passés 24 h sous la peau du lapin, a donné des résultats très encourageants soit pour le cancer mammaire des souris R III, soit pour les souris AkR leucémiques[1], enfin ces mêmes techniques furent appliquées au cancer chez l'homme.

Nous avons analysé avant et après passage sur lapin les protéines et les acides nucléiques de divers tissus de souris et humains, normaux et cancéreux, pour mieux connaître nos implants néoplastiques. L'importance des acides nucléiques dans la cancérogenèse est généralement admise; notamment LATARJET et al.[2] sont parvenus à produire des cancers multiples chez la souris par administration d'acides nucléiques extraits de tissus leucémiques.

Matériel et méthodes. Les analyses électrophorétiques[3] et immunoélectrophorétiques[4] ont été effectuées sur lames de verre gélosées en milieu tampon véronal (pH = 8,6; $\mu = 0{,}06$; 6 volts/cm). La durée a été de 2 h pour les protéines (1 h pour l'immunoélectrophorèse) et de 30 min pour les acides nucléiques.

Les analyses électrophorétiques des protéines ont été effectuées soit directement sur le tissu (0,5 à 2 mg), soit après extraction à + 4° (5 à 40 mg) par un tampon phosphate 0,02 M, pH = 7,3 (coloration: Amidosschwarz).

Les acides nucléiques[5] examinés également après électrophorèse, ont été extraits selon la méthode de SCHUSTER et al.[6] sur 5 à 20 mg de tissu frais (coloration: vert de methyl-pyronine). Leur concentration a été appréciée d'après une échelle étalon effectuée avec un acide nucléique

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xtrait de la levure (provenance Mann Research Lab. New-'ork) (Fig. 1). Toutes ces analyses ont été faites dans des)nditions de concentration identiques, soit donc compa-ables entre elles

Résultats. 1. Tissus de souris. Nous avons examiné les rotéines et les acides nucléiques de tissus de souris nor-hales, AkR (leucémiques) et d'une souris R III (tumeur ammaire). Pour chaque animal nous avons étudié: le œur, les muscles striés, les reins, la rate, le foie, le thymus, s surrénales, le cerveau, les ganglions lymphatiques et ucémiques (souris AkR) de même que les tumeurs mam-aires (souris R III).

Toutes les expériences ont été faites sur des tissus non ésanguinés. Nous avons constaté dans tous les tissus vant passage sur lapin des fractions protéiques non écelées dans le sérum de souris; ces tissus tant des souris ormales que AkR et R III ont donné comparativement ans ces trois groupes, des images très proches. Après assage, il y a diminution des protéines du type tissulaire, image se rapprochant de celle du sérum de lapin, avec pendant en général une fraction albuminique légèrement lus rapide.

La concentration des acides nucléiques décelés avant assage a varié considérablement selon les tissus (Fig. 2). Après passage de 24 h sur lapin les acides nucléiques des ssus présentent des spots d'intensité faible et comparable, qui représente une forte diminution de ceux-ci dans mines et de γ-globulines humaines. Dans le cas d'un glioblastome nous avons constaté, après passage, une ligne immunoélectrophorétique dans la zone des préalbumines.

Les acides nucléiques extraits des divers tissus examinés ont tous donné des spots très nets. Après passage sous la peau du lapin, nous avons fait les mêmes constatations que pour les tissus de souris. Dans un cas de carcinome du sein, la diminution des acides nucléiques initiaux a été d'environ 65% alors qu'elle a été d'environ 90% pour un glioblastome.

Les analyses des tissus lyophilisés avant et après passage sur lapin ont donné des résultats comparables aux tissus frais.

Fig. 1

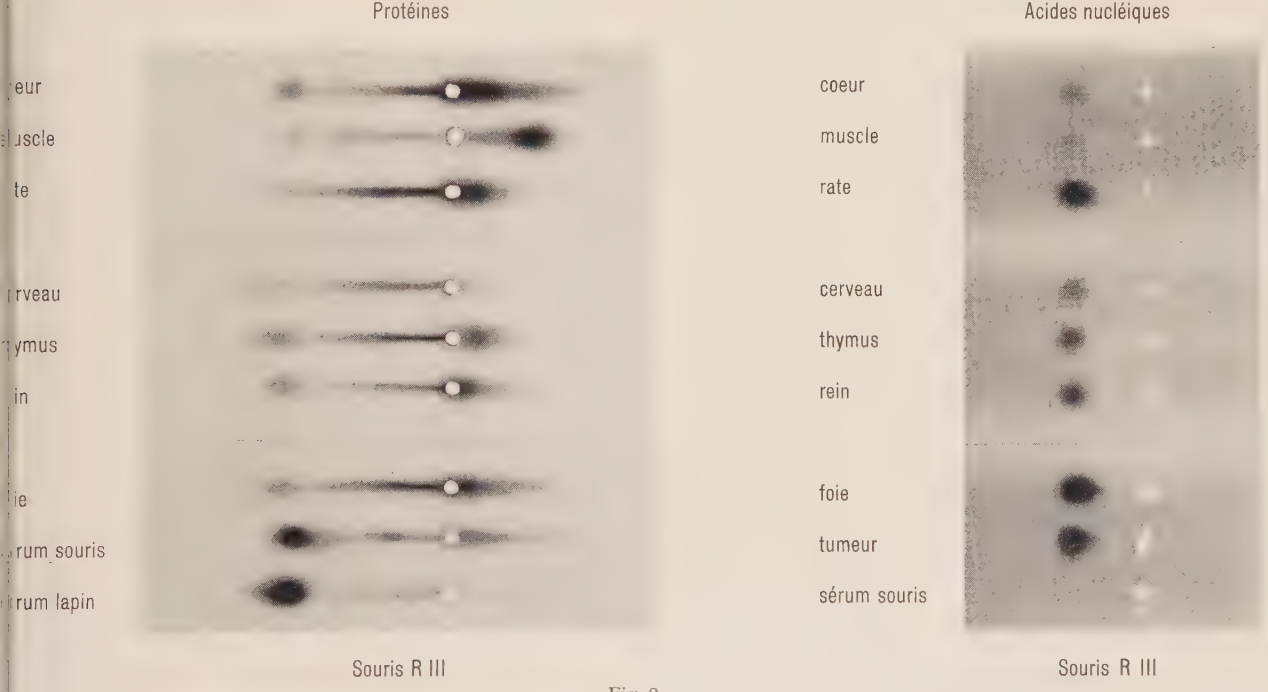

Fig. 2

s tissus riches avant passage (95% de perte pour les gan-ions leucémiques AkR). Par contre il y a peu ou pas de erte dans les tissus initialement pauvres en acides ucléiques (Fig. 3).

2. Tissus humains. Nous avons examiné plusieurs pes de tissus humains néoplastiques. Lors de leur assage sous la peau du lapin, il y a diminution progressive es protéines humaines, celle-ci étant fonction du temps e passage, de la forme et de la texture du tissu. Nous avons vérifié par immunoélectrophorèse, méthode qui rès 24 h de passage décelait encore la présence d'albu-

Summary. Cancerous or leukotic tissue fragments modified by a 24 h subcutaneous implantation in the rabbit, become capable of favouring the inhibition of cancerogenesis.

This paper is concerned with the study of proteins and nucleic acids of the so-treated implants.

[7] Qu'il nous soit permis de remercier ici Madame Docteur Glarner pour l'aide précieuse qu'elle nous a apportée en pratiquant les passages sous la peau du lapin.

Fig. 3

R. Fischer[7] et J. Monnier

Centre de Transfusion Sanguine, Hôpital Cantonal, Genève (Suisse), le 29 novembre 1960.

Beitrag zur Papierchromatographie der Phytoöstrogene

Im Anschluss an die erfolgreichen chromatographischen Arbeiten von Bose und Chandran[1], Biggers und Curnow[2], Lyman et al.[3] wird in der vorliegenden Mitteilung die papierchromatographische Trennung von Phytoöstrogenen aus Pflanzenextrakten beschrieben.

Das Pflanzenmaterial wurde jeweils mit 96% Alkohol extrahiert, der Alkohol unter vermindertem Druck abdestilliert und das Residuum in 1–2 ml Azeton gelöst. Von dieser Lösung wurden Proben in Form eines 2 mm breiten und 4 cm langen Streifens auf Papier Whatman Nr. 2 und 4 aufgetragen. Als Entwickler wurde eine Mischung von 2 Teilen Chloroform, 1 Teil konzentrierter Essigsäure und 1 Teil Wasser benutzt, und zwar in zwei Modifikationen: 1. untere Schicht dieser Mischung, 2. obere Schicht.

Die Chromatogramme wurden luftgetrocknet und die Stoffe mit UV-Licht und 0,1% Ferrichlorid-Lösung nachgewiesen. Die östrogene Aktivität der nachgewiesenen Stoffe konnte nach vorheriger Extraktion mit Methanol an infantilen Mäuseweibchen von 6–8 g bestimmt werden (Methodik der Bestimmung wie in der früheren Arbeit Churý[4]).

Die untere Schicht der Entwicklermischung ist für die Trennung der Stoffe nicht geeignet, da das Chlorophyll mitgerissen und die Stoffe maskiert werden. Bei der Anwendung der oberen Schicht bleibt das Chlorophyll am Start zurück, was eine gute Trennung der anwesenden Stoffe ermöglicht. Im UV-Licht bei Anwendung von Kobaltglasfilter können mindestens 4, höchstens 8 Streifen sichtbar werden, deren Rf-Werte in der Tabelle I angeführt sind.

Die Ergebnisse der geprüften biologischen Aktivität der in den einzelnen Streifen anwesenden Stoffe sind der Tabelle II zu entnehmen:

Es ergibt sich, dass man chromatographisch auch solche Spuren von sexualaktiven Stoffen im Pflanzenmaterial nachweisen kann, die sonst in gereinigten Auszügen mittels biologischer Tests nicht erfassbar sind.

Den Detektionsergebnissen mit 0,1% Ferrichlorid-Lösung nach, sind die Stoffe in den Streifen mit Rf 0,95 bis 0,97 wahrscheinlich von Flavonoiden-Charakter. Ob der blau fluoreszierende Stoff (Rf 0,49) mit dem Cumestrol identisch ist, müssen spektrophotometrische Messungen beantworten. Beim hellblau fluoreszierenden Stoff (Rf 0,85) nehmen wir an, dass es sich um eine wahrscheinlich noch unbekannte sexualaktive Substanz handelt, zu deren näherer Charakterisierung Versuche im Gange sind.

Résumé. Description d'une méthode chromatographique sur papier pour la séparation des phytoestrogènes dans les extraits non purifiés de plantes. En utilisant la couche supérieure d'un mélange de deux parts de chloroforme, d'une part d'acide acétique et d'une part d'eau distillée, on a extrait 3 substances, qui, soumises au test biologique sur des souris infantiles, se montrèrent estrogènes.

J. Churý

Biologisches Institut der Veterinärmedizinischen Fakultät Brno (CSR), 30. Juni 1960.

Tabelle I

Streifen	Fluoreszenz	Rf-Wert	Bemerkung
I	rot	—	—
II	hellblau	0,37	—
III	blau	0,49	aktiv
IV	dunkel	0,65	—
V	grün	0,76	—
VI	hellblau	0,85	aktiv
VII	grünlichgelb	0,94	aktiv
VIII	gelb-grün	0,97	aktiv

Tabelle II

Fluoreszenz	mg Uterusgewicht	% Zuwachs gegen Kontrolle
rot	3,7 ± 0,35	—
blau	10,7 ± 0,80	+ 163%
hellblau	13,00 ± 3,00	+ 242%
grünlich-gelb	6,50 ± 0,30	+ 71%
Kontrolle	3,80 ± 0,40	—

[1] J. L. Bose und K. Chandran, J. Sci. industr. Res. *14C*, 128 (1955).
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[3] R. L. Lyman *et al.*, Arch. Biochem. Biophys. *80*, 61 (1959).
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Further Studies on the Influence of Chloride on the Renal Tubular Reabsorption of Bicarbonate in the Dog

The influence of NaCl-induced rises in plasma chloride concentration (P_{Cl}) on tubular bicarbonate reabsorption was studied during $NaHCO_3$ loading 1. in normal dogs, 2. in respiratory acidotic dogs and 3. in dogs treated with acetazolamide[1]. The data of these experiments were compared with the results previously obtained in this laboratory in normochloremic[2] and in hypochloremic[3–5] animals.

[1] Diamox was generously supplied by the Lederle Laboratories.
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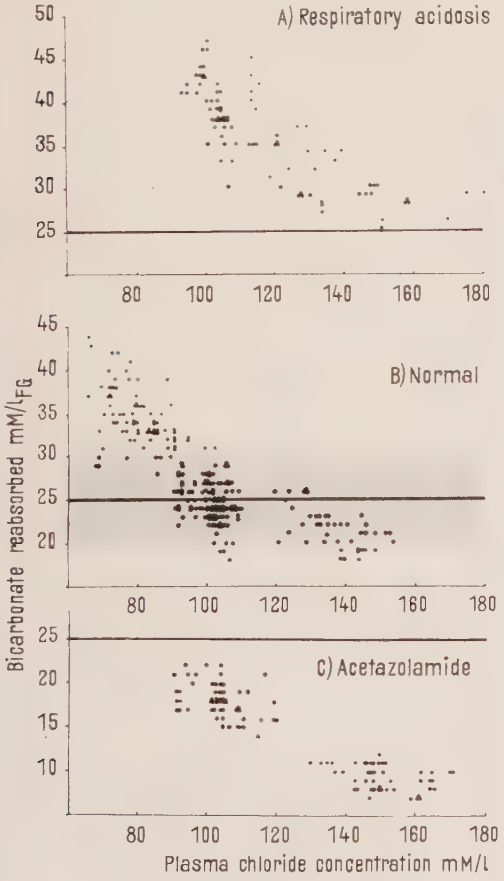
Experimental procedures. 1. *Normal dogs.* Bicarbonate reabsorption rate ($BHCO_3R$) was measured a) at normal P_{Cl} values (29 experiments on 17 dogs, 134 clearance periods), b) at high P_{Cl} values (8 experiments on 7 dogs, 43 clearance periods) and c) at low P_{Cl} values (13 experiments on 10 dogs, 84 clearance periods).

2. *Respiratory acidotic dogs.* While blood pCO_2 values were maintained between 132 and 164 mm Hg by the inhalation of CO_2 20% in O_2, $BHCO_3R$ was measured a) at normal P_{Cl} values (11 experiments on 9 dogs, 43 clearance periods) and b) at high P_{Cl} values (10 experiments on 9 dogs, 45 clearance periods).

3. *Acetazolamide-treated dogs:* During continuous infusion of acetazolamide (30–55 mg/kg/h, preceded by a priming dose of 10 mg/kg), $BHCO_3R$ was measured a) at normal P_{Cl} values (8 experiments on 7 dogs, 65 clearance periods) and b) at high P_{Cl} values (5 experiments on 5 dogs, 46 clearance periods).

Results. 1. *Normal dogs* (Fig. B). The respective values of the slope of the regression lines calculated for the relation between $BHCO_3R$ and P_{Cl} in the 3 groups of normal animals are: 0.295 in hypochloremia, 0.167 in normochloremia and 0.130 in hyperchloremia. Thus, the effect of Cl on $BHCO_3R$ is progressively diminishing as P_{Cl} values are increasing so that the relation between $BHCO_3R$ and P_{Cl} is truly a curvilinear one.



Bicarbonate reabsorption rate values plotted with plasma chloride concentration values in respiratory acidosis A, in normal dogs B, and during acetazolamide treatment C.
The horizontal lines drawn at $BHCO_3R$ values of 25 mM/L_{GF} represent the mean value of $BHCO_3R$ during $NaHCO_3$ infusion in normal dogs. The hatched zone in B represents the 4 σ range of the mean value of $BHCO_3R$.

2. *Respiratory acidotic dogs* (Fig. A). As for the normal animals, the depression induced by Cl on $BHCO_3R$ is of greater magnitude at normal than at high P_{Cl} levels: the values of the slope of the regression lines calculated for the relation between $BHCO_3R$ and P_{Cl} are 0.276 for the normochloremic, and 0.169 for the hyperchloremic respiratory acidotic animals.

3. *Acetazolamide-treated dogs* (Fig. C). Despite the profound inhibition of $BHCO_3R$ produced by acetazolamide, this tubular transport is further depressed (to the extremely low level of 7.0 mM/L_{GF}) by NaCl administration. As was noted for normal and for respiratory acidotic animals, the influence of Cl on $BHCO_3R$ is diminishing as P_{Cl} is progressively rising: the regression line has a slope of 0.114 at normal, instead of 0.055 at high P_{Cl} levels. Both values differ significantly from zero ($P < 0.001$).

Comment. Although the depression of $BHCO_3R$ produced by Cl was previously noted in normal[6] and in respiratory acidotic[7] dogs, our data further show that the magnitude of the influence of hyperchloremia is primarily affected by the initial level of $BHCO_3R$ itself. This phenomenon is demonstrated by the fact that, for any given value of P_{Cl} increment, the intensity of Cl influence on $BHCO_3R$ is diminishing in the following order: respiratory acidotic, normal and acetazolamide-treated animals. The values of the slope of the regression lines calculated for the relation between $BHCO_3R$ and P_{Cl} in the three conditions investigated, differ significantly in the sole case of respiratory acidotic versus acetazolamide-treated animals ($0.02 < P < 0.05$ in normochloremia and $0.001 < P < 0.01$ in hyperchloremia).

Finally, our data on respiratory acidotic dogs loaded with $NaHCO_3$ alone demonstrate that, contrarily to a recently held view[8], the increase in $BHCO_3R$ observed at high plasma bicarbonate concentration values is readily explained by the spontaneous reduction in P_{Cl} which follows acute $NaHCO_3$ loading.

Résumé. L'intensité de la dépression qu'entraîne l'hyperchlorémie sur la réabsorption tubulaire du bicarbonate chez le chien paraît être essentiellement déterminée par le niveau initial de la réabsorption du bicarbonate.

Ch. Toussaint and P. Vereerstraeten

Laboratoire de Médecine expérimentale, Université libre de Bruxelles et Fondation médicale Reine Elisabeth, Bruxelles (Belgium), October 14, 1960.

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[7] J. G. Hilton, N. E. Capeci, G. T. Kiss, O. R. Kruesi, V. V. Glaviano, and R. Wegria, J. clin. Invest. *35*, 481 (1956).
[8] W. B. Schwartz, A. Falbriard, and G. Lemieux, J. clin. Invest. *38*, 939 (1959).

Effects of Stimulation and Inhibition of Bicarbonate Reabsorption on the Renal Tubular Transport of Sodium and Chloride in the Dog

The numerous observations[1–3] demonstrating the influence of changes in plasma chloride concentration (P_{Cl}) on tubular bicarbonate reabsorption rate ($BHCO_3R$) have led us to investigate the tubular transport of Na and Cl in

[1] R. F. Pitts and W. D. Lotspeich, Amer. J. Physiol. *147*, 138 (1946).
[2] J. G. Hilton, N. E. Capeci, G. T. Kiss, O. R. Kruesi, V. V. Glaviano, and R. Wegria, J. clin. Invest. *35*, 481 (1956).
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dogs submitted to two experimental procedures affecting primarily $BHCO_3R$: acute respiratory acidosis and acetazolamide treatment.

Experimental procedures. In each experiment, hypertonic NaCl was infused to raise P_{Cl} and P_{Na} to 170 and 210 mM/l, respectively.

1. *Respiratory acidosis* (17 experiments performed on 16 dogs, 94 clearance periods): respiratory acidosis (blood pCO2 134–174 mm Hg) was maintained throughout each experiment by the inhalation of CO_2 20% in O_2. In 7 experiments, P_{BHCO_3} ranged from 19.5 to 24.7 mM/l so that $BHCO_3$ excretion rate was negligible. In 10 experiments, P_{BHCO_3} was maintained between 27.5 and 45.0 mM/l by adding $NaHCO_3$ to the saline infusion, so that $BHCO_3$ excretion rate exceeded 5.0 mM/l glomerular filtrate in 26 clearance periods.

2. *Acetazolamide treatment* (6 experiments performed on 6 dogs, 49 clearance periods): acetazolamide[4] was infused throughout each experiment at a rate of 30–45 mg/kg/h, after a priming dose of 10 mg/kg. P_{BHCO_3} ranged from 10.6 to 22.3 mM/l and $BHCO_3$ excretion rate exceeded 5.0 mM/l glomerular filtrate in 38 clearance periods.

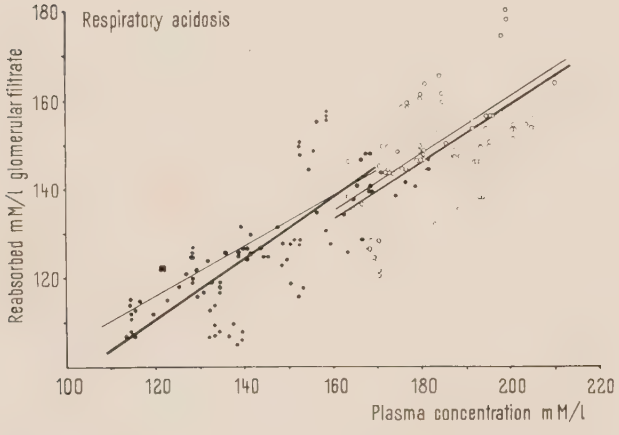

Fig. 1. Relation between Cl (black circles) and Na (open circles) reabsorption rate and plasma concentration values during respiratory acidosis. The thick line represents regression during acidosis and the thin line regression previously calculated for normal dogs.

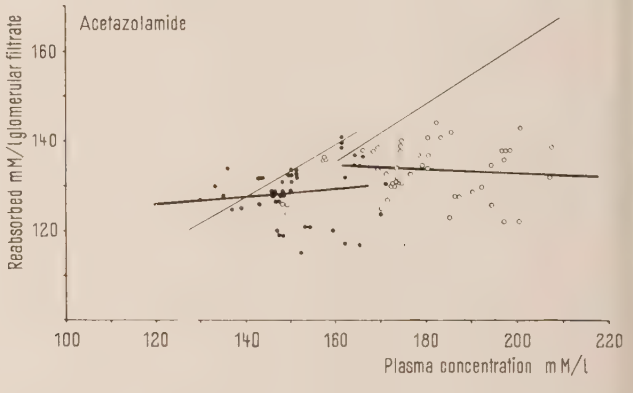

Fig. 2. Relation between Cl (black circles) and Na (open circles) reabsorption rate and plasma concentration values during acetazolamide infusion. The thick line represents regression during acetazolamide treatment, and the thin line regression previously calculated for normal dogs.

Results. 1. *Respiratory acidosis.* In Figure 1, the values of Na and Cl reabsorption rate have been plotted with the corresponding values of P_{Na} and P_{Cl}. It is obvious that NaR and ClR rise linearly with P_{Na} and P_{Cl}. The slopes of the regression lines calculated for the relation between reabsorption rate and plasma concentration values are 0.655 for Na and 0.674 for Cl. Both values are significantly different from zero, but they do not differ from the slopes calculated in normal dogs[5]. Increase in P_{BHCO_3} during respiratory acidosis did not affect the relation between NaR and P_{Na} or between ClR and P_{Cl}.

2. *Acetazolamide treatment.* Figure 2 presents in a similar manner the data obtained in acetazolamide-treated dogs. The values of the slope of the regression lines calculated for the relation between reabsorption rate and plasma concentration values are – 0.030 for Na and 0.089 for Cl. These values are not significantly different from zero, but they differ from the slopes obtained in normal dogs[5].

Comment. The lack of stimulation of respiratory acidosis on ClR in our experiments is in marked opposition with the findings of WESSON[6] in similar experiments. It should be noted that, in the dog[7] as well as in the rat[8,9] submitted to prolonged CO_2 exposure, without NaCl loading, Cl excretion rate has been regularly found to be increased during the first two days of CO_2 exposure. Our negative findings in acute respiratory NaCl-loaded dogs would suppress one of the objections raised against PITTS's theory[10] which assumes a primary role to active Na transport in the genesis of an electrochemical potential responsible for the passive reabsorption of $BHCO_3$ and Cl.

Acetazolamide treatment markedly reduces NaR and ClR. Similar results were obtained by SCHWARTZ and RELMAN[11] and by WESSON[12], although smaller doses of acetazolamide are devoided of any significant effect[12]. This lack of action of small doses on ClR, despite their effect on $BHCO_3R$, affords evidence, but no proof, that the chloruretic effect of large doses is independent of the action of the drug on hydrogen secretion. Such an interpretation would stand against the second objection opposed to PITTS's theory[10], for, according to this conception, if the action of acetazolamide on ClR was dependent on $BHCO_3R$ inhibition, ClR would be increased rather than decreased by the drug.

Résumé. L'acidose respiratoire aiguë n'affecte pas significativement la réabsorption tubulaire du Na et du Cl, alors que celle-ci est déprimée par l'administration d'acétazolamide.

CH. TOUSSAINT and P. VEREERSTRAETEN

Laboratoire de Médecine expérimentale, Université libre de Bruxelles et Fondation médicale Reine Elisabeth, Bruxelles (Belgium), November 3, 1960.

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Crossed Reflex Actions by Low Threshold Muscle Afferents

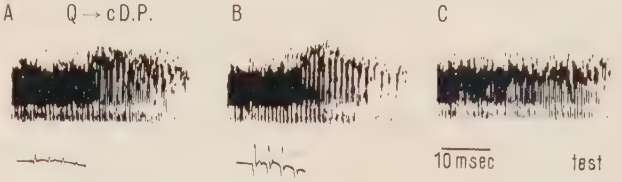
A number of crossed spinal reflex actions are known to exist[1] but there have been few attempts to analyse them in terms of the contributions from various somatic afferent systems. Crossed effects by volleys in high threshold muscle afferents have been reported[2] and PERL has described certain actions by single volleys in the large muscle afferents (group I)[3]. He found that a group I volley from knee flexors or extensors evokes inhibition followed by facilitation in motoneurones of corresponding contralateral muscles and, less consistently, a reciprocal pattern in motoneurones of their antagonists. Crossed effects on adequate activations of muscle receptors were assumed to be mediated by group I afferents[4].

Cats made spinal by high lumbar section about two weeks before the final experiment, were used in the present investigation, because it was found that crossed actions from low threshold afferents were easiest elicited in these preparations. Even so, single conditioning maximal group I volleys hardly ever influenced contralateral monosynaptic test reflexes (whereas volleys in high threshold muscle afferents had pronounced effects). On the other hand, if a train of conditioning volleys were given, considerable contralateral group I effects were observed. This is illustrated in the Figure where the monosynaptic test reflex from the DP (deep peroneal = *tibialis anterior + extensor digitorum longus*) nerve is conditioned by a train of group I volleys from the contralateral quadriceps nerve. Each record consists of many (about 50) superimposed traces of monosynaptic reflex discharges scattered across the screen, while the conditioning stimuli are given at a fixed interval after the sweep start. The unconditioned monosynaptic test discharge is shown alone in record C. A clearcut crossed action appears already at a stimulus strength of 1.7 times threshold (A) and the action increases when the stimulus strength is raised to 2.75 times threshold (B), which is just submaximal group I. In excitable preparations, it was often sufficient to use two conditioning volleys to evoke crossed actions, otherwise 3–6 were needed. By increasing the number of conditioning stimuli the effect increased markedly. On repetitive stimulation at constant strength the successive volleys gradually decreased. When high range group I stimulation was used, monophasic recording of the incoming volley from dissected dorsal root-filaments was often made afterwards; and it was proved that the effects observed were evoked at stimulus strengths subliminal to group II fibres. In special control experiments it was established that at frequencies used there was no temporal summation of subthreshold stimuli in group II fibres, in other words a train of stimuli did not activate fibres not activated by the first stimulus.

In the Figure it should also be noted that a stimulus activating only about one fifth of the group I fibres was sufficient to evoke crossed actions (A). Such low threshold activation was also frequently observed between extensors. The stimulus strength was regularly increased with fine gradations which resulted merely in an increased effect. There was never any evidence of dual crossed group I actions (cf. Figure where records A and B are chosen from a series with increasing stimulus strength).

The most striking and constant feature of the crossed group I pattern is the prominent excitatory action between extensors (*quadriceps, gastrocnemius-soleus, plantaris* and *flexor digitorum longus*). There was never any inhibitory action from extensors to any contralateral extensor nucleus. From flexors investigated, facilitation was found from DP to contralateral extensor nuclei, while BSt (*posterior biceps-semitendinosus*) seldom evoked any group I effect, but inhibition was observed once to quadriceps.

As regards the receptiveness of flexor nuclei, there were differences between BSt and DP. Group I volleys from extensors usually facilitated DP, whereas actions to BSt were less frequent. Between flexors group I, facilitation from DP to DP was observed but never any effect from BSt to BSt.



Upper records consist of superimposed traces of many monosynaptic reflex discharges evoked from the nerve to the deep peroneal muscles (*tibialis anterior + extensor digitorum longus*) and recorded in the ventral root. At each sweep the reflex discharge is automatically delayed so as to traverse the screen and this procedure is repeated 4 times. The distribution of reflex discharges is not uniform, the density decreases in the end of the record. The unconditioned test reflexes are shown in C, and in A and B the actions of a train of conditioning volleys from the contralateral quadriceps nerve evoked at a stimulus strength of 1.7 respectively 2.75 times threshold for the nerve. Lower traces show the conditioning volleys from the quadriceps nerve recorded in the dorsal root entry zone.

To summarize. The use of repetitive stimulation has proved valuable for disclosing crossed group I connections and a description of their pattern has been given. On the other hand, with repetitive stimulation it is more difficult to separate contributions from the fast and slow component of the group I volley, and so far it has not been possible to differentiate conclusively between actions evoked from large muscle spindle afferents and Golgi tendon organ afferents.

Zusammenfassung. Bei chronisch spinalen Katzen wurde mit Hilfe tetanischer Reizung festgestellt, dass grosse afferente, von Muskeln kommende Fasern gekreuzte Wirkungen auf monosynaptische Reflexe ausüben. Die Ausdehnung der Effekte wird beschrieben.

BIRGITTA HOLMQVIST

Department of Physiology, University of Lund (Sweden), October 12, 1960.

[1] C. S. SHERRINGTON, Quart. J. exp. Physiol. *2*, 109 (1909).
[2] E. R. PERL, J. Neurophysiol. *21*, 101 (1958). – B. HOLMQVIST, Exper. *16*, 459 (1960).
[3] E. R. PERL, J. Neurophysiol. *21*, 101 (1958).
[4] E. R. PERL, J. Physiol. *145*, 193 (1959).

On the Mechanism of Increasing the Tuberculostatic Activity of Isoniazid in the Presence of 4-Aminobenzoic Acid

We have shown in previous communications[1,2] that 4-aminobenzoic acid (PABA) increased *in vitro* the bacteriotropic activity of isoniazid on *M. tuberculosis*, whereas 2-aminobenzoic acid exerted an exactly opposite influence[3].

[1] R. URBANČÍK, Amer. Rev. Tuberc. *76*, 706 (1957).
[2] R. URBANČÍK, Amer. Rev. Tuberc. *78*, 802 (1958).
[3] Z. ŠIMÁNĚ and R. URBANČÍK, Exper. *16*, 148 (1960).

The mechanism by which PABA increased the activity of isonizid is still unknown. It was, however, suggested by KUBALA[4] that this might have been explained by competitive acetylation of PABA instead of isoniazid, which was known to operate *in vivo*[5] and undoubtedly could have occurred also in the conditions *in vitro*.

The reliability of this hypothese was first checked in an experiment where the synergistic influence of both PABA and 4-acetamido-benzoic acid (Ac-PABA) on the isoniazid activity was compared.

The strains *Mycobacterium 607* and H 37 Rv were maintained by serial passages on the surface of the Proskauer-Beck synthetic liquid medium without albumine or serum. This medium (pH 6.8) was distributed in 50.0 ml amounts in 250 ml Erlenmeyer flasks, to which isoniazid alone or with either PABA or Ac-PABA was added, and seeded with one loop of a 7-days old culture of the *M. 607* strain or a 14-days old culture of the H 37 Rv strain. Isoniazid was sinter-sterilized and added aseptically to the medium, PABA and Ac-PABA were added to the medium before autoclaving. All experiments were run in four parallel flasks. After 7 days incubation at 37°C for the *M. 607* strain or 14 days incubation for the H 37 Rv strain all cultures were autoclaved and the bacterial mass was separated by filtration from the medium. Further description of the method may be found elsewhere[1]. Concentrations of the drugs as well as results of the experiments are summarized in Table I and II.

Table I

Drug	Concentration in μg/ml	Dry weight of bacteria in g[a, b]
Control	—	0.5424
PABA	200	0.5290
Ac-PABA	260[c]	0.5513
Isoniazid	1	0.0692
Isoniazid + PABA	1 + 200	0.0316
Isoniazid + Ac-PABA	1 + 260	0.0888

[a] Strain *Mycobacterium 607*.
[b] Mean value from four parallel flasks.
[c] Equimolar amount to 200 μg of PABA.

Table II

Drug	Concentration in μg/ml	Dry weight of bacteria in g[a, b]
Control	—	0.2933
PABA	200	0.2849
Ac-PABA	260[c]	0.2906
Isoniazid	0.05	0.0581
Isoniazid + PABA	0.05 + 200	0.0333
Isoniazid + Ac-PABA	0.05 + 260	0.0599

[a] Strain H37Rv.
[b] Mean value from four parallel flasks.
[c] Equimolar amount to 200 μg of PABA.

It may be seen from both Tables that, in both the *M. 607* or the H 37 Rv strain, Ac-PABA was ineffective in increasing the tuberculostatic properties of isoniazid, whereas PABA showed the previously described synergistic influence on the isonizid activity. These results appear to offer some evidence in favour of the afore mentioned competitive acetylation hypothesis. Nevertheless, a direct proof of acetylation of either isoniazid or PABA by mycobacterial cultures *in vitro* was definitely needed. It is difficult to trace acetylated isoniazid under the conditions of our experiments. We attempted therefore to prove the formation of Ac-PABA from PABA in mycobacterial cultures *in vitro*.

The Proskauer-Beck medium (pH 6.8), to which 200.0 μg of PABA per ml were added before autoclaving was distributed in Erlenmeyer flasks and seeded as described above. After incubation for the afore-mentioned time intervals, the cultures of both strains were autoclaved and the bacterial mass was separated by filtration from the medium. The filtrates, each time from a control medium and from the medium with PABA, were further investigated as to their content of Ac-PABA by paper chromatography. The chromatograms were run ascendently on No. 3 Whatman paper with use of *n*-propanol, water, and ammonia solvent (50 : 38 : 12). Ac-PABA was hydrolysed on the paper by spraying with 10% hydrochloric acid and exposing it for 10 min over boiling water. The spots were detected by dipping the paper into the Ehrlich reagent with acetone[6].

Our attempts to demonstrate Ac-PABA in culture filtrates of both strains remained, however, unsuccessfull. Thus we may conclude that under the conditions of our experiment PABA is not acetylated by either strain *in vitro*, and the competitive acetylation hypothesis of increasing the bacteriotropic effect of isoniazid in the presence of PABA can hardly be maintained.

Our experiments, however, have shown that Ac-PABA does not increase the effect of isoniazid on either of the strains used. Thus we may suggest that *in vivo*, where acetylation of PABA undoubtedly takes place, the combination of isoniazid and PABA will probably be no more effective than isoniazid alone, except perhaps with very high doses of PABA. This suggestion has been verified[7].

Zusammenfassung. 4-Acetaminobenzoesäure hat *in vitro* im Unterschied zu 4-Aminobenzoesäure, keinen günstigen Einfluss auf die antituberkulotische Wirkung von Isoniazid. Bei den Stämmen *Mycobacterium 607* und H 37 Rv wurde jedoch chromatographisch keine Bildung acetylierter 4-Aminobenzoesäure nachgewiesen.

R. URBANČÍK, P. KRAUS, and Z. ŠIMÁNĚ

Tuberculosis Research Institute, Prague-Bulovka (ČSR), September 28, 1960.

[4] E. KUBALA, Amer. Rev. Tuberc. *78*, 949 (1958).
[5] J. C. BELL, D. K. RIEMENSNIDER, and R. S. MITCHELL, Amer. Rev. Tuberc. *76*, 152 (1957).
[6] J. B. JEPSON, Lancet *ii*, 1009 (1955).
[7] R. URBANČÍK, Z. ŠIMÁNĚ, P. KRAUS, and I. REIL, Exper. *17*, 84 (1961).

Isoniazid and 4-Aminobenzoic Acid in the Treatment of Murine Tuberculosis[1]

As shown previously, 4-aminobenzoic acid (PABA) increases the tuberculostatic activity of isoniazid *in vitro*[2]. It was now of interest to investigate the effect of isoniazid combined with PABA in the conditions *in vivo*.

[1] Part of these experiments was performed in the Institute of Microbiology, University of Brno.
[2] R. URBANČÍK, Amer. Rev. Tuberc. *76*, 706 (1957).

No. of mice	Group	Dosage	ST_{50} [a]	99% confidence interval [b]
12	Control	— [c]	19.4	
10	PABA	0.5 mg	16.5	
13	INH	100 μg	36.0	$33.04 < \mu < 38.96$
11	INH + PABA	100 μg + 0.5 mg	38.2	$35.58 < \mu < 40.78$
13	Control	—	13.1	
11	PABA	1.0 mg	10.3	
12	INH	100 μg	30.6	$22.11 < \mu < 39.21$
12	INH + PABA	100 μg + 1.0 mg	29.6	$21.28 < \mu < 37.88$
13	Control	—	15.6	
10	PABA	3.0 mg	8.9	
15	INH	100 μg	22.2	$18.15 < \mu < 26.11$
14	INH + PABA	100 μg + 3.0 mg	30.9	$28.26 < \mu < 33.58$

[a] Median survival time expressed in days.
[b] Calculated with use of the *t*-test and expressed in days.
[c] To all mice in all control groups plain saline was administered daily substaneously for 14 days.

White mice of the 'H' strain, weighing 16 to 20 g were infected intravenously with 0.5 ml of a 10-days old Dubos culture of the H37Rv strain of *M. tuberculosis*. The animals were always distributed by random selection into groups of at least 10 mice and were fed in the course of the treatment with rolls and water *ad libitum*. The therapy was started the day after the infection and lasted 14 days. Isoniazid and PABA[3] were administered subcutaneously, each of them separately in one daily dose. Three sets of experiments were performed at different time intervals, each of them with a different dose of PABA. The median survival time was registered; the results, which were analysed with use of the *t*-test[4], are summarized in the Table.

It may be seen that with 0.5 mg or 1.0 mg of PABA no increase could be observed in the therapeutic activity of isoniazid, if compared with the therapeutic effect of isoniazid alone. There is, however, a statistically significant difference in the therapeutic activity of isoniazid combined with 3.0 mg of PABA, if compared with isoniazid alone.

The mechanism of this phenomenon is not clear. It has been suggested that this increase in activity of isoniazid in the presence of PABA might be attributed to the competitive acetylation of PABA instead of isoniazid, which is known to operate *in vivo*[5], but could not be proved to take place *in vitro*[6]. There are, however, some suggestions that PABA cannot be acetylated by tuberculous animals[7]. Thus this question still remains open.

As discussed in a previous communication[8], PABA decreases the median survival time of tuberculous mice. This phenomenon, for which we still have no explanation, may readily be observed in the Table presented.

Zusammenfassung. Bei der Mäusetuberkulose war die kombinierte Therapie von Isoniazid mit 4-Aminobenzoesäure (PABA) (3 mg/Tag/Tier) wirksamer als diejenige von Isoniazid allein. Bei niedrigerer PABA-Dosis wurde der Synergismus nicht nachgewiesen.

PABA allein verkürzt die mittlere Überlebenszeit bei tuberkulösen Mäusen.

R. Urbančík, Z. Šimáně, P. Kraus, and I. Reil

Tuberculosis Research Institute, Prague-Bulovka (ČSR), September 29, 1960.

[3] Sodium salt of PABA, produced as 'Paraminan'® by R. Gallier, Paris.
[4] E. Weber, *Grundriss der biologischen Statistik* (G. Fischer, Jena 1956), p. 158.
[5] J. C. Bell, D. K. Riemensnider, and R. S. Mitchell, Amer. Rev. Tuberc. *76*, 152 (1957).
[6] R. Urbančík, P. Kraus, and Z. Šimáně, Exper. *17*, 83 (1961).
[7] S. P. Martin, S. N. Chaudhuri, C. D. Cooper, and R. Green, Ciba Foundation Symposium on Experimental Tuberculosis (1955), p. 102.
[8] R. Urbančík, Jap. J. Tuberc. *6*, 95 (1958).

Über die Bindung von Kallidin an Heparin

Nach früheren Beobachtungen [1,2] bildet Heparin mit Histamin eine nichtdialysierbare Verbindung, in der die beiden Stoffe mit grösster Wahrscheinlichkeit auch in den Mastzellen gespeichert sind[3]. Wir haben nun festgestellt, dass auch das Polypeptid Kallidin, das chemisch und pharmakologisch von Bradykinin nicht unterscheidbar ist[4], mit Heparin[5] einen undialysierbaren Komplex bildet und wie Histamin durch die Verbindung 48/80 wieder freigesetzt werden kann. Der Kallidin-Heparin-Komplex ist auch aus sehr verdünnter Lösung durch Alkohol fällbar.

Bei der stehenden Dialyse wässeriger Kallidinlösungen durch Cellophanmembranen (Volumenverhältnis Innen- zu Aussendialysat zum Beispiel 1:4) findet man unter unseren Versuchsbedingungen[6] auch in Abwesenheit von Heparin nach 24 h nicht wie erwartet 80% Kallidin aussen und 20% innen, sondern nur etwa 10% aussen und 0% innen. Das Kallidin ist nicht zerstört, sondern an die Cellophanmembran adsorbiert und kann durch Elution der zerschnittenen Membran mit n-NH_3 wiedergewonnen werden.

In Gegenwart von Heparin (bis zur unteren Grenze von 2,5 IE Heparin pro 1 Einheit Kallidin) findet sich die gesamte eingesetzte Kallidinmenge im Innendialysat. Dies besagt, dass die Dissoziationskonstante der Kallidin-Heparin-Verbindung äusserst niedrig sein muss, weil andernfalls nach einigen Stunden das Kallidin trotz der Gegenwart von Heparin an die Cellophanmembran adsorbiert sein müsste. Da die biologische Aktivität des Kallidins in seiner Bindung an Heparin nicht beeinträchtigt ist, erfolgt die Anlagerung des Kallidins so, dass zwar die Diffusion, nicht aber die Anlagerung an den Rezeptor der Uterus- oder Darmwand behindert ist. Das durch Heparin gebundene Kallidin ist nur in geringem Masse gegen den Angriff der Kallidinase geschützt. Bei Anwesenheit von Heparin und Verbindung 48/80 (Mengenverhältnis zum Beispiel 30 γ Verbindung 48/80 auf 20 IE Heparin) verhält sich Kallidin im Dialyseversuch wie für freies Kallidin geschildert.

Versuchsbeispiel zur Fällung von Kallidin durch Heparin: 100 Einheiten Kallidin + 1000 Einheiten Heparin gelöst in je 1 ml Wasser + 8 ml 96%iger Alkohol. Der sofort

[1] R. Amann und E. Werle, Klin. Wschr. *34*, 207 (1956).
[2] E. Werle und R. Amann, Klin. Wschr. *34*, 624 (1956).
[3] A. Schauer und E. Werle, Z. ges. exp. Med. *131*, 100 (1959).
[4] M. Schachter, in *Polypeptides which Affect Smooth Muscles and Blood Vessels* (Pergamon Press, London 1960), p. 199.
[5] Verwendet wurden Heparin-Novo (5000 IE/ml) und Heparin Hoffmann-La Roche in Substanz (113 IE/mg).
[6] (10–20 Kallidin-Einheiten in ca. 5 ml). 1 Einheit Kallidin ist diejenige Kallidinmenge, die man aus 1 ml Rinderplasma mit 1 Einheit Kallikrein aus Schweine-Submaxillarisdrüsen bei 20°C freisetzen kann.
[7] R. Jaques, K. Küttner, H. J. Bein und R. Meier, Exper. *16*, 147 (1960).
[8] R. Amann und E. Werle, Klin. Wschr. *35*, 22 (1957).

sich bildende weisse Niederschlag wird abzentrifugiert und in wenig Wasser aufgenommen. Die Lösung enthält rund 90% der eingesetzten Kallidinmenge. Diese empfindliche Fällungsreaktion dürfte auch für die Isolierung von Kallidin von praktischer Bedeutung sein.

JAQUES et al.[7] haben kürzlich festgestellt, dass auch Hypertensin durch Heparin gebunden wird. Da ausser Histamin, Hypertensin und Kallidin anscheinend auch Thrombin[8] mit Heparin einen Komplex bildet, nehmen wir mit JAQUES et al. an, dass der Bildung von Heparin-Komplexen eine allgemeinere Bedeutung zukommt.

Summary. Kallidin gives, with heparin, a non-dialysable complex, which can be precipitated with alcohol. In this binding, kallidin is pharmacologically active, but may be split off by the substance 48/80.

E. WERLE, I. TRAUTSCHOLD und G. LEYSATH

Klinisch-chemisches Institut an der chirurgischen Klinik der Universität München (Deutschland), 21. Oktober 1960.

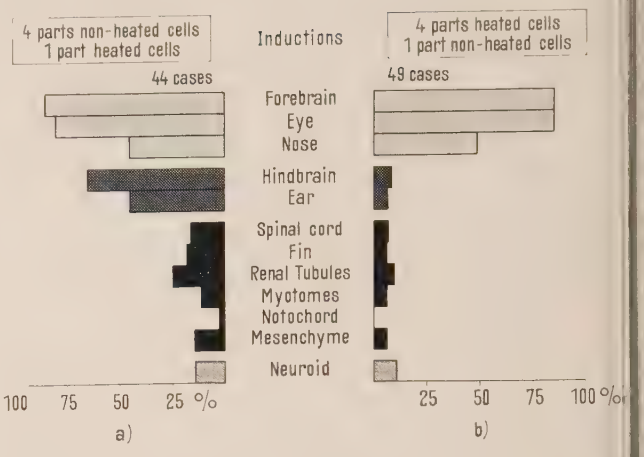
Quantitative Evidence for the Two-Gradient Hypothesis in the Primary Induction

The idea of a two-gradient system acting in the primary induction, presented by LEHMANN[1] and YAMADA[2], had earlier led us to experiments in which two heterogeneous tissues of qualitatively different inductive action were implanted simultaneously in order to test their combined effect[3]. A new type of induction was observed, explained as being a combined action of two principles, one a mesodermalizing and the other a neuralizing inductive system. A very similar theory was simultaneously presented by YAMADA and TAKATA[4].

These ideas were based on two types of experiment: The combination of two different inductors, or a separation of the two different inductive effects by chemical treatment of one inductor tissue, originally 'combined'. None of these experiments, however, could give us quantitative information on the hypothetical principles operating in the primary induction. In such a quantitative operation, our present methods with their marked limitations seem to offer only two possible ways of approach; either the size of the implant is varied, or different concentrations of the active factors incorporated in some non-inducing material are used. For a number of reasons, both of these methods have to be considered inadequate, and also the techniques give rise to a variety of technical errors. Another set-up was accordingly planned so as to get at least some relative data on the amount of the different inducing principles, and the significance of their ratio to the induction process.

Method. HeLa-cells cultured in a medium containing human serum were used as inductors[5]. After mechanical removal of the cells from the walls of the culture flasks, they were treated as follows:

From 10 to 11 million cells were collected from 3 culture flasks, washed several times with saline solution, and finally dispersed in 10 cm^3 of saline. Of this homogeneous cell suspension, 5 cm^3 was heated on a waterbath for 30 min at 65°—a treatment which is known to inactivate the mesodermalizing capacity of cells and tissues[5,6]. Meanwhile, the other half of the suspension was kept in an incubator at 37°C. Following this, the two suspensions were combined in two test tubes in the following ratios: A) 1 cm^3 heated cells + 4 cm^3 non-heated cells; B) 4 cm^3 heated cells + 1 cm^3 non-heated cells.



Percentage of the induced structures in the experimental series.

During the whole of this procedure, every care was taken to keep the cell suspensions dispersed homogeneously, and the tubes were repeatedly shaken between the different stages of treatment. The two combined suspensions (A, B) were then simultaneously centrifuged at 5000 rpm, and the compact sediment covered with cold 70% alcohol. After 4 to 6 h of alcohol treatment, the cells were washed and used in explantation experiments according to 'sandwich' method[7]. The presumptive epidermis of young Triturus vulgaris-gastrulae was used, and the explants were cultivated for 10 days in Holtfreter-solution. The differentiations were analyzed microscopically from serial sections.

Results. The results are demonstrated in the Figure. Only one comment has to be made as regards the Figure. As was the case in our earlier works, only the presence or absence of the different induced structures was recorded and thus the analysis gives no information on the amount or size of the secondary formations. For instance, the large masses of striated muscle seen in some cases in series A were recorded as being equal to the explants in series B where often only a few muscle fibers were found. Thus, in this respect the title of our report is misleading, and the analysis of the inductions is by no means quantitative.

Discussion. In comparison with our earlier findings obtained with HeLa-cells in implantation experiments[5], the low frequency of mesodermal structures was conspicuous in both of the present series. This is partly correlated with the generally known fact that mesodermalization is always weaker in explantation experiments than in those of implantation[3,8]. Furthermore, we know from our earlier and partly unpublished experiments that a maximal mesodermalizing effect is noted in HeLa-cells, only if their culture medium is changed shortly before the experiment.

[1] F. E. LEHMANN, Rev. Suisse Zool. *57*, Suppl. 141 (1950).
[2] T. YAMADA, Embryologia *1*, 1 (1950).
[3] S. TOIVONEN and L. SAXÉN, Ann. Acad. Sci. Fenn. A. IV *30*, 1 (1955).
[4] T. YAMADA and K. TAKATA, J. exp. Zool. *128*, 291 (1955).
[5] L. SAXÉN and S. TOIVONEN, J. Embryol. exp. Morphol. *6*, 616 (1958).
[6] S. TOIVONEN, J. Embryol. exp. Morphol. *2*, 239 (1954).
[7] J. HOLTFRETER, Arch. exp. Zellforsch. *15*, 281 (1934).
[8] H.-H. CHUANG, Roux' Arch. *139*, 556 (1939).
[9] A. DALCQ, in *Fundamental Aspects of Normal and Malignant Growth* (W. W. Nowinski, Ed., Elsevier Publ. Co., New York 1960), p. 305.

[10] The investigation was supported by research grants from the Finnish State and the Sigrid Juselius Fund.

In the present experiments, the cells were grown for three days in the same culture medium. Accordingly, the results can hardly be compared with our earlier observations[5], and a comparison will be made only between the two present series, A and B.

By reason of the small number of mesodermal differentiations in both series, the difference is not very striking, but the greater number of all spinocaudal structures in series A can be noted. The most striking difference is in the number (and size) of the deuterencephalic structures. According to our two-gradient hypothesis[3], the deuterencephalic structures are developed as a result of an initial stimulus of two inductive principles, the neuralizing and the mesodermalizing principle. Furthermore, we know that the mesodermalizing factor(s) is inactivated by heat treatment, and is thus not active in the heated HeLa-cells[5,6]. The hindbrain structures seen in some cases of series B must consequently be a result of a neural induction together with the mesodermalizing effect of the small amount of non-heated HeLa-cells in the implant. When the relative amount of the mesodermalizing factor(s) is increased by changing the ratio of heated and non-heated cells in the inductor, there appears a definite increase in the number (and size) of the deuterencephalic structures (series A). We are therefore inclined to conclude that the regional type of induction is here not dependent only on the presence or absence of certain inductive principles, but that it is also a reflection of the relative amounts of these active factors.

Our modification of the two-gradient hypothesis has recently been criticized and considered as an 'oversimplification'[9]. We agree in that it is a simplified model, but we should like to stress that it is only a working hypothesis, and not intended to form an explanation of the most complicated process of primary induction. So far, it seems to explain the earlier findings on relations between the inducing principles and the induced structures.

The quantitative experiments will be continued employing different ratios of heated and non-heated cells, and in the presentation of these results the problem will be discussed in more detail.

Zusammenfassung. Die Einwirkung quantitativer Unterschiede induzierender Agenzien wurde untersucht. HeLa-Zellen, mit und ohne Wärmebehandlung, wurden in zwei verschiedenen Proportionen (4:1 bzw. 1:4) gemischt und als Induktor in Epidermisexplantaten des Molcheimes benutzt. Das Resultat (Fig.) zeigt, dass der Induktor, der mehr Zellen ohne Wärmebehandlung enthält, mehr mesodermale, aber auch mehr deuterencephale Gebilde induziert. Das Resultat wird mit der Zwei-Gradienten-Hypothese der induzierenden Agenzien erklärt.

S. Toivonen[10], L. Saxén, and T. Vainio

Department of physiological Zoology, University of Helsinki (Finland), November 8, 1960.

An Experimentally Produced Change in the Sequence of Neuralizing and Mesodermalizing Inductive Actions

According to Nieuwkoop et al.[1], the regionality of the central nervous system is caused by two successive stimuli. The first of these leads to the non-specific 'activation' of the competent ectoderm, which reacts, differentiating to the archencephalon and its derivatives only. The second stimulus then causes the 'transformation' of the 'activated' ectoderm, resulting in a modification of the archencephalic differentiation tendencies in structures typical of more caudal regions of the nervous system. Nieuwkoop states that the 'transformation', i.e. the differentiation of more caudal formations of the central nervous system, occurs only if the ectoderm is previously 'activated'.

In our opinion[2,3], two different principles are active in the primary induction, neuralizing (N), and mesodermalizing (M). The N principle, if it acts alone, causes the differentiation of the archencephalon and its derivatives. The M principle correspondingly brings about the mesodermal differentiations, if acting alone. But, in the normogenesis, these two principles form two partly overlapping gradients. This system would direct the regionality of all the structures induced.

I have shown earlier[3], in experiments with heterogenous inductors, that under experimental conditions the mesodermal differentiations can be caused with no previous neuralizing action ('activation' of Nieuwkoop). In order to test the independent characteristics of these two principles assumed by us, I arranged experiments in which an attempt was made to change the normal sequence of the actions of these two principles.

Material and methods. The 'sandwich' method was used. The host epidermis was from young gastrulae of the common newt (*Triturus vulgaris*). The inductor materials were marrow from the thigh-bone of the guinea-pig, and HeLA cells[4] cultured *in vitro*. The bone marrow tissues was fixed in 70% alcohol for 24–72 h, and washed in sterile saline for 1–2 h before use in experiments. The HeLa cells were cultured in Roux flasks in a growth medium consisting of 30% pooled fresh human serum, and 70% Hanks' solution[5]. The cells adhering to the walls of the Roux flasks were mechanically detached without trypsinization, centrifuged, washed three times in buffered saline, and heated in glass tubes for 30 min at 70°C in a water bath. Subsequently, the cells were fixed in alcohol and washed in sterile saline in the same way as the bone marrow mentioned above. The Holtfreter saline for culturing the explants was buffered with phosphates[6]. The explants were cultured for 10 days. In experimental series B and C (Fig.) the explants were opened, and the inductor was removed after cultivation for 3 h. In series C, the bone marrow tissue was replaced with HeLa cells. The explants were fixed in Bouin's fluid, first stained in bulk with borax-carmine, and the serial sections counter-stained with picro-blue-black.

Results. A detailed analysis of the structures induced and the arrangements for the experiments is given in the Figure.

Series A and D are controls which show the inductive actions of both of the inductors used. The bone marrow induced almost mesodermal structures. The spinal cord was the only neural formation induced. It was always short and thin, and on the tip of the induced tail only. The heated HeLa cells were strictly pure inductors of archencephalic structures.

If the bone marrow was removed after 3 h (B), the explant differentiated to all the types of differentiations as in series A, but less frequently. The lower frequency of the

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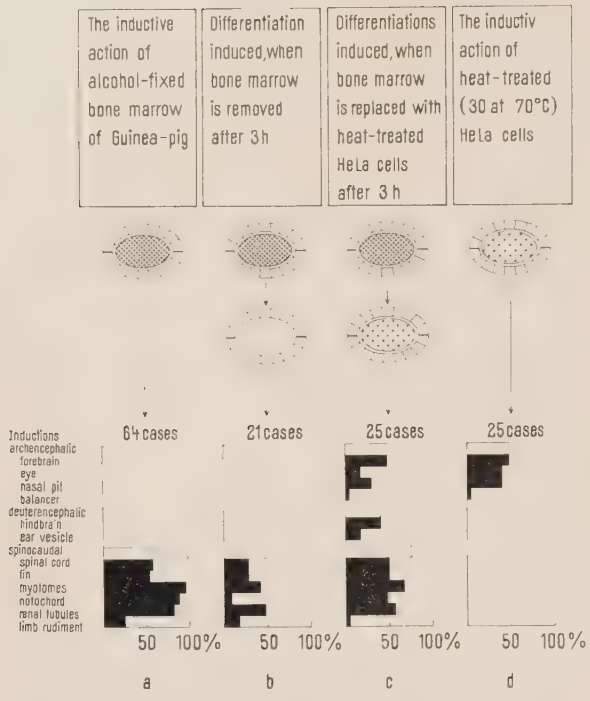

The arrangement of the experiment and the analysis of the structures induced in the different series.

differentiations compared with the control series (A) might in part have depended on the fact that the inductor tissue was somewhat infiltrated with living cells of the host, and, when the inductor was removed, some of the determined cells were removed with it.

Series B is also a control for the 3 h action of the bone marrow in series c, where it was replaced with the heated HeLa cells. In this series, deuterencephalic differentiations were also noted, although they were lacking completely in the other series. One has to conclude that they were induced by a simultaneous action of both of the inductors, which were separately unable to induce them, a fact which we have noted earlier[2]. Another fact one might consider in this connection is that the differentiations of the mesodermal structure in series C were more frequent than in the corresponding control (B). The fact that the mesodermal differentiation is stimulated by the presence of neural structures, has been observed earlier[7,8].

Discussion. The above results corroborate our earlier opinion that the mesodermalizing and the neuralizing inductive actions are caused by different principles, because their normal sequence can be experimentally reversed, that is, to use the terminology of Nieuwkoop, there may occur a mesodermalizing 'activation' and neuralizing 'transformation'.

As a matter of fact, it has to be admitted that the difference between the views of Nieuwkoop et al.[1] on the one hand, and of myself and my co-worker[2] on the other, is largely a terminological one, as I have pointed out earlier[3]. Nieuwkoop's non-specific 'activation' process corresponds to the action of our N principle, and his 'transformation' process appears to correspond to the action of our M principle. But, as these two actions can be separated one from the other, as the specific actions of the inductors used in the experiments above indicate, and as the sequence of these two actions can be reversed, in my opinion the terminology of Nieuwkoop is no longer quite adequate.

For the present, it seems that our two-gradient-hypothesis[2], which is similar to that presented by Lehmann[9] and Yamada[10], provides a satisfactory explanation of the regionality caused by the action system in the primary induction process.

Zusammenfassung. Die normale Reihenfolge der neuralisierenden und mesodermalisierenden Aktionen wurde experimentell umgekehrt. Im Explantatversuch diente die präsumptive Epidermis des Molchkeimes als Reaktionsmaterial. Als neuralisierender Induktor wurden HeLa-Zellen nach Wärme- und Alkoholbehandlung, und als mesodermalisierender das Knochenmarkgewebe des Meerschweinchens nach Alkoholbehandlung benutzt. Die Anordnung der Versuche sowie die Analyse der induzierten Gebilde sind in der Figur dargestellt. Das Resultat wird mit der Zwei-Gradienten-Hypothese der induzierenden Agenzien erklärt.

S. Toivonen[11]

Department of physiological Zoology, University of Helsinki (Finland), November 8, 1960.

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First Results of Tissue Culture in *Drosophila*

It is well known that it is possible, after 4–5 days culture, to achieve *in vitro* differentiation of imaginal discs of the eyes and wings of *Drosophila melanogaster* (Demal[1], Gottschewski[2], Grace[3], Horikawa[4–7], Horikawa and Sugahara[8], Kuroda[9–11], Kuroda and Yagamuchi[12], Kuroda and Tamura[13]). Horikawa and Sugahara[8] kept in a culture for 72 h also salivary glands, testicles, fat bodies and cephalic complexes, while Horikawa and Kuroda[14] succeeded in obtaining an active multiplication of the haemolymph cells for two weeks.

Undifferentiated tissue of the larval central nervous system and cells obtained from the lymph gland seemed to us to be the most suitable material for further attempts while experimenting with various types of culture medium. The basic elements that we used in the composition of the culture media were: plasma of heparinized cock's blood (P); extract of chick embryo after 8–9 days' incubation (EE); synthetic medium for insects according to Grace (Paul[15]) without cholesterol (SM); extract of *D. melanogaster* larvae of the wild Varese or Aspra 52 strains (ELV, ELA). Antibiotics were added to the cultures.

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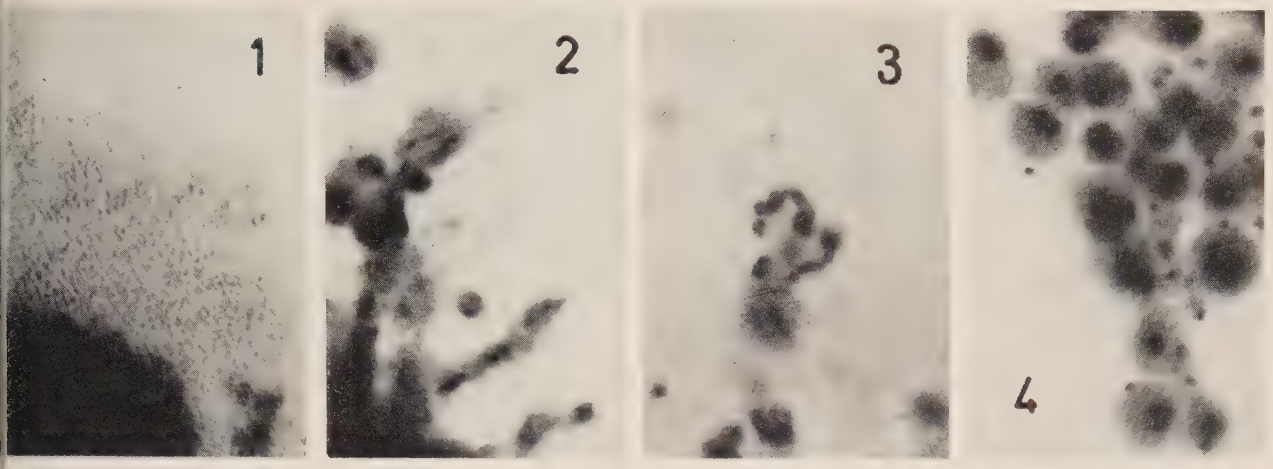

Fig. 1. Cell migration from a Varese ganglion (9th day) in phase contrast (× 140). – Fig. 2. Cell migration from a Varese ganglion (19th day) stained with Delafield's haematoxylin (× 1400). – Fig. 3. Mitosis in Aspra ganglion (17th day) stained with orcein acetic (× 1650). – Fig. 4. Cells from a lymph gland of S. Maria strain (10th day) stained with May Grümwald-Giemsa (× 1200).

In the experiments with embryo extract, Waddington's physiological solution (pH 7.3) was used, while in the experiments with synthetic medium we preferred to use the same solution of salts that makes up part of the medium itself (pH 6.6). The cultures were prepared by the hanging drop method, preserved in a thermostat at 25°C ± 0.5 and transplanted every 7 days.

The best results were obtained with the cephalic ganglia (2 cerebral hemispheres and ventral ganglion), and are summarized in the Table.

The condition of the cultures was judged on the basis of observation both by phase contrast microscope and of material fixed in Carnoy and stained with Delafield's haematoxylin. Mitoses were observed also on crushed material stained with acetic orcein.

It is evident that we managed to obtain survivals of 4–5 weeks, although the optimum period is limited to between 14 and 21 days. In fact it is during the second week of culture that the spreading of cells outside the ganglion is noted (Fig. 1 and 2), at the same time as the appearance of some mitotic figures, especially in Aspra larvae ganglia (Fig. 3). The latter tend to form more globous and thicker expansions in comparison with Varese or S. Maria larvae ganglia.

With regard to the results obtained with a culture medium composition that can be said complete (P + EE + + EL or P + SM + EL), survival is reduced noticeably in the absence of larvae extract, and made impossible with only embryo extract or only synthetic medium. The lack of plasma, on the other hand, brings about a precocious lysis of the ganglion membrane with consequent liberation of the cells in the culture medium. This phenomenon is particularly evident in the synthetic medium. In this case, we succeeded in obtaining the survival of isolated cells for 39 days: however no mitosis was seen. The inability of isolated cells to multiply is justified by a similar fact already noted in mammals (Sato, Fisher and Puck[16]). In fact it was observed how the same culture medium which was sufficient for organs or tissue fragments had to be further enriched with certain substances in order to cultivate various types of isolated cells.

A similar explanation may be given of the results obtained by cultivating lymph glands of the S. Maria strain. This gland is particularly fragile and allows the isolated cells to escape easily. The latter survive in the culture media experimented by us for 10–13 days without mitoses being observed (Fig. 4). Sometimes they take on an ameboid aspect.

Stocks	Culture medium	Age of cultures in days 7	14	21	28	35
I 1. Varese – S. Maria	P + EE + ELV	+	++	++	+–	
2. Aspra	P + EE + ELV	+	++			
3. Aspra	P + EE + ELA	+	++			
II 4. Varese – S. Maria	P + SM + ELV	+	++	++	+–	
5. Aspra	P + SM + ELV	+	++	++	++	+–
6. Aspra	P + SM + ELA	+	++	++	+–	
III 7. Varese – S. Maria	P + SM	++	++	+–		
8. Aspra	P + SM	++	++	+–		
IV 9. Varese – S. Maria	EE + ELV	+	++			
10. Aspra	SM + ELA	++	+++	+++	++	+– ++–
11. Varese – S. Maria	P + ELV	––				

\+ = compact ganglion – living cells.
+· = ganglion with expansions – living cells pyknotic nuclei and mitoses.
+– = ganglion with expansions – cultures only partially living
–– = dead ganglion
+++ = dissolved ganglion – living free cells
++– = free cells – partially living culture.
––– = dead free cells.

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The lymph glands of Varese strain, with compact structures, survive for an even shorter length of time; thereafter part of the cells undergo processes of turnig black, which are similar to those that transform haemolymph cells into pseudotumours.

In this respect, it has been seen that the large haemolymph cells (CASTIGLIONI[17]), which have already aggregated to form tumours (Aspra 52 strain) and are kept in a culture for 15 days, are subject to progressive melanization. Also other workers, who have tried to cultivate melanotic tumours, have observed a progressive expansion of melanization in cells near the tumour (KURODA and TAMURA[18–25]) or in cells which have migrated into other tissues from the tumour itself (FRIEDMAN and BURTON[26], FRIEDMAN et al.[27]) without this proving that there was any cellular multiplication. In our experiments, in one culture alone was a bridge formed of living cells (some probably in mitosis) between two fragments of tumours.

While admitting the preliminary nature of the present research, we believe we can point, as being of some interest, to the results obtained by us up to date, especially those on ganglia, both for the relatively long survival period and for the documented growth through mitotic divisions. This preliminary research will allow us next to try to obtain clone cultures on culture media kept under the strictest control.

Riassunto. Gli autori riescono ad ottenere la sopravvivenza in cultura di gangli cefalici larvali di *Drosophila melanogaster* per oltre un mese, con mitosi durante la 2ª e 3ª settimana, mentre le ghiandole della linfa si mantengono vive solo per 10–13 giorni.

M. C. CASTIGLIONI and
G. REZZONICO RAIMONDI

Istituto di Genetica, Università di Milano (Italy), October 24, 1960.

Stretching Activity in Dogs Intracisternally Injected with a Synthetic Melanocyte-Stimulating Hexapeptide

In dogs, intracisternal injections of highly purified ACTH preparations induce typical, prolonged, and repeated stretching crises[1,2]. Similar effects are caused by purified MSH preparations[3] and by solutions of ACTH made with NaOH *N*/10 treated at 100°C for 20 min[2]. These findings suggest that the chemical groups eliciting the stretching responses are related with those stimulating melanocytes. This correlation is supported by results obtained with dogs given intracisternally the acetate salt of the synthetic hexapeptide H Glu(NH_2)-His-Phe-Arg-Try-Gly-OH with MSH-like activity. 750 γ/kg or less of this peptide (given intracisternally) evoke stretching responses similar to those following injections of MSH or ACTH. 1.5 mg/kg of the peptide causes lasting depression and scialorrhea but not a stretching crisis.

The melanocyte stimulating effect of the peptide is 2×10^5 U/g[4]; that of a purified MSH preparation (732179 A by Armour Laboratories, Chicago, Ill.) 5×10^3 U/g. The threshold dose for the stretching response was respectively 500 and 5 γ/kg: indeed these two different pharmacological actions are strictly related. The hexapeptide, but not MSH, shows a paradoxycal behaviour and a pharmacological response evoked by the polypeptide may change qualitatively with respect to the dose given intracisternally.

The Figure illustrates a typical stretching crisis in a dog injected intracisternally with the hexapeptide.

A typical stretching crisis in a dog injected intracisternally with the melanocyte-stimulating hexapeptide

Riassunto. La iniezione endocisternale nel cane di dosi fino a 0.75 mg/kg del sale acetico dell'esapeptide H.Glu(NH_2)-Ist-Fen-Arg-Tript-Gli.OH, svolgente un'attività melanoforo-stimolante della intensità di 2×10^5 U/g, induce delle tipiche crisi di stiramento, del tutto simili a quelle che si ottengono iniettando per la stessa via dell'ACTH o dell'MSH. Dosi di 1.5 mg/kg invece non inducono più crisi di stiramento ma deprimono notevolmente gli animali e determinano una intensa scialorrea. Sembra di poter ammettere che la struttura chimica responsabile dell'effetto sui melanociti sia anche responsabile dell'induzione delle crisi di stiramento.

W. FERRARI[5], G. L. GESSA, and L. VARGIU

Istituto di Farmacologia, Università degli Studi, Cagliari (Italy), July 13, 1960.

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The Effect of Parabiosis and Fluid Restriction on the Development of Azo Dye Induced Rat Liver Tumors[1]

CAMPBELL and STONE[2] have shown that slices of liver tumor synthesize 1/3 to 1/2 the amount of serum albumin produced by slices of normal liver. GLINOS[3] has provided evidence that depletion of the plasma protein level by plasmapheresis can accelerate liver regeneration in the rat and that fluid restriction retards liver regeneration by increasing the relative concentration of plasma proteins.

[1] This research was supported by a grant from the National Science Foundation (6139) and an institutional grant from the American Cancer Society.
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The present experiments attempt to increase the plasma protein level in rats fed an azo dye and to determine the effect upon the incidence and rate of liver tumor formation.

Materials and methods. Rats with developing liver tumors were united in parabiosis with normal rats, or they were united on each side to normal rats as parabiotic triplets. The normal rats might be expected to provide plasma proteins to the pre-tumorous rats by way of the common vascular connections. Fluid restriction, during and after feeding of the dye, was also employed with some of the rats. Serum density determinations were made according to the method of Van Slyke described in TÁRNOKY[4]. The rats were fed 3'-methyl-4-dimethylamino-azobenzene for three months at a 0.06% level in the synthetic diet developed by GELBOIN, MILLER and MILLER[5]. Control rats of the same weight were fed the synthetic diet without the carcinogenic azo dye. Parabiosis was carried out according to the method of HILL[6].

Results. Parabiosis. The average survival period of 21 dye-fed control rats was 5.5 weeks after the cessation of dye feeding. Twelve dye-fed rats were united in parabiosis with single control rats; the average survival time was 7 weeks after termination of feeding of the dye. Control rats which had been in parabiotic union with tumorous rats until their death were separated surgically and kept under observation. Laparotomies upon these rats did not reveal any sign of liver damage or tumor formation 2.5 months after separation from the tumorous rats. In eight cases dye-fed rats were united between two normal rats as parabiotic triplets. The average survival time of the tumorous rats was 10.8 weeks after dye feeding was discontinued.

Fluid restriction. Ten rats were subjected to five day periods of fluid restriction during the three months the dye was fed and for $1^1/_2$ months after dye feeding. Ten other rats of a similar age were given water *ad libitum* but the consumption of the synthetic diet containing the azo dye, and later the rat chow, of each of these rats was limited so that it was similar to that of the fluid restricted rats. Another group of ten rats had access to the diet and water at all times. $1^1/_2$ months after the dye feeding period the average weight of the fluid restricted rats was 184 g, that of the pair-fed controls was 178 g and the average weight of the rats with unlimited food and water supply was 340 g. All the rats were sacrificed at this time and it was found that none of the fluid restricted or pair fed rats showed any sign of liver damage or tumors while the group without fluid or food restriction all showed varying degrees of liver tumor development.

Before the above groups of rats were sacrificed serum density determinations[4] were performed on the rats of the fluid and food restricted groups, as well as upon five normal rats and five normal rats that had been subjected to two seven day periods of water restriction. The average serum density of both types of normal rats and the fluid restricted rats was 1.029 or 8% serum protein, whereas the average serum density of the food restricted rats was 1.027 or 7.5% serum protein.

Discussion. In the present experiments parabiosis (with one or two normal rats) slightly retards the average rate of tumor development in rats which had been fed the azo dye, but does not prevent their eventual death from liver tumors. Since the fluid restriction experiments demonstrated that tumor development could be inhibited by decreasing the food consumption after the period of dye feeding, it seems probable that the slight retardation in tumor development due to parabiosis is attributable to decreased food consumption by the dye-fed rats because of the difficulties of feeding while in the parabiotic association.

Prolonged periods of fluid restriction were found to inhibit liver tumor formation in rats fed the azo dye. However pair-feeding experiments indicated that tumor development was inhibited due to the decreased food consumption of these rats. This conclusion is further supported by the finding that fluid restriction did not increase the relative concentration of serum proteins. TANNENBAUM and SILVERSTONE[7] have previously found that the promotional, but not the initiation, of carcinogenesis can be inhibited by caloric restriction.

Résumé. Chez le rat, le développement des tumeurs du foie est ralenti lorsqu'on met les individus atteints en parabiose avec des individus normaux et arrêté si l'on réduit leur ration d'eau. Mais il semble que dans les deux cas, ces effets ont pour cause déterminante la diminution de la nourriture consommée.

R. FLICKINGER

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Human Vascular Antigen Complement Consumption Test of Hypertensive Patients (Preliminary Report)

Owing to its high incidence throughout the world, hypertensive vascular disease is constantly in the focus of interest. Recent research has greatly enhanced our knowledge of the condition, but the pathogenesis has still many obscure features.

We have undertaken to study vascular lesions in hypertensive patients, with special reference to the potential role of autoagressive processes. To elucidate the problem, complement consumption tests have been made with vascular antigens, thus modifying the 'leucocyte complement consumption test' of CHUDOMEL, JEZKOVA, and LIBÁNSKY[1] so that it became suitable for the demonstration of binding of vascular antigen-autoantibodies.

Methods. Specimens of arteries and aorta were taken from various parts of blood group '0' hypertensive cadavers, homogenized in 'Ultra-turax' and stored at $-10°C$. To titrated amounts of the homogenate, active test sera were added and the mixtures were incubated at 37°C. After 1 h of incubation and centrifugation, the sera were tested for complement content. A decrease of serum complement results (as compared with the serum complement level in the absence of vascular antigen) if a vascular antigen-autoantibody union has taken place. Corresponding to the measure of the fall in complement titre, we speak about a '+' reaction (3 tubes), '++' (5 tubes) and '+++' reaction (5–10 tubes). A difference of 0–2 tubes may be accepted as negative. According to CHUDOMEL, JEZKOVA, and LIBÁNSKY[1], the method is equally suitable for the demonstration of complete and incomplete antibodies.

Results. The complement consumption tests yielded positive results in 60 out of 122 cases of hypertension, equivalent to 49.1% of all cases. In 3 cases, the sera were

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	Number of patients examined	Number of positive results by the complement consumption test	Number of negative results by the complement consumption test	Number of anti-complementary sera
Sera from normal individuals	41	3	38	—
Sera from patients with various internal diseases	44	7	37	—
Sera from patients with hypertensive disease	122	60	59	3

anticomplementary. The controls were normal males and females, with normal blood pressure. Of the 41 normal controls, 3 showed positive test (7.3%) as compared with 7 out of 44 patients suffering from various internal diseases (16.1%). Thus, the results indicate that, although not specific, the vascular antigen complement consumption test employed by us was more often positive in the cases of hypertension than in the normal controls or among the patients with various internal diseases. The complement fixation tests involving the use of tissue antigens, the non-specific reactions that may occur, the difficulties that may be encountered, as well as the theoretical considerations, have been discussed in detail by Bakos, Schulhof, Szilárd, and Vajda[2], who showed that a positive complement fixation with tissue antigens was more common among patients with polyarthritis than among those suffering from various internal disorders, and that in some cases also an organ-specificity should be taken into account.

The mechanism of the positive complement consumption tests in cases of hypertension is still unclear. It appears that the binding of an autoantigen-autoantibody is involved. This view is supported by the earlier experiments of Goreczky, Róth, Sárfy, and Sümegi[3], who showed that in animals the pathomorphological changes of periarteritis nodosa could be produced by treatment with arteriotoxic serum. These data were confirmed years later by Stefanini[4]. Szigeti and Jákó[5] demonstrated, by the antihuman globulin consumption method of Steffen[6], the presence of incomplete antibodies in the sera from rabbits immunized with homologous vascular wall antigen. It was remarkable in the clinical evaluation of our positive cases that most of the patients had shown the symptoms and signs of renal lesion. Autoagression is well-known to play a role in renal diseases (Cavelti[7], Frick[8], Sarre and Rother[9], Vorlaender[10], Wendlberger and Fröhlich[11], Fröhlich and Pressinger[12].

In the light of these data from the literature, the results of the vascular antigen complement consumption tests, as well as the presence of vascular lesions in patients with hypertension, call attention to a potential role of autoagression in a certain percentage of such cases. It is still unknown how these processes of autoagressive nature begin and how they influence hypertension.

Zusammenfassung. Der «Gefässantigentest» (complement-consumption-test) wurde bei 122 hypertonischen Kranken, bei 44 verschiedenen internen Patienten und bei 41 gesunden Personen durchgeführt. Das Ergebnis war bei den Hypertonikern in 49,1%, bei den Internen in 16,1% und bei den Gesunden in 7,3% positiv. Das Verfahren kann aber nicht als spezifisch betrachtet werden, obwohl bei den Hypertonikern das Ergebnis öfters als bei anderen Patienten positiv ist.

K. Köröskényi,
F. Juba, and Gy. Vajda

MÁV Hospital and Policlinic, Budapest (Hungary), May 16, 1960.

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Development of Digits from the Proximal Pre-Axial Material of the Wing and Hind-Limb Bud in Chick Embryos

The epidermis plus a fairly thick layer of the underlying mesenchyme removed from the proximal two-thirds of the pre-axial border of one limb bud (St. 22 to 25 chicken embryos) was transplanted on a shallow incision of the distal third of the dorsal aspect of the same (A) or of the heteronymous (B) limb bud. The graft lay parallel to the preserved apical ridge of the host bud (Fig. *inset*). At the time of the operation the structure of the epidermis of the graft is similar to that of the epithelial covering of the dorsal and ventral surfaces of the limb bud; a small part of the *apical ridge* proper is present only in the grafts taken from stage 22 not from stage 23 to 25 embryos.

In the normal development *in situ*, the material which is transplanted in these experiments would give rise to a part of the territory of the arm and forearm (and respectively of the thigth and leg). Actually, from one to three supernumerary digits developed from the heterotopically grafted material of the pre-axial border. The characters of these digits are in the great majority of the cases typical of the donor limb (Fig.).

The results mentioned bear some resemblance to those obtained by implanting small isolates from the thigh mesoderm under the *apical ridge* of the wing bud (Saunders et al.[1]), or by transplanting a semilunar pre-axial portion of the wing bud over the proximal stump of the hind-limb bud (Case CH, St. 22, Hampé[2]). However, in our experiments, the heterotopically grafted mesenchymal precursor of the arm and forearm (and respectively of the thigh and leg) was not in contact with the normal apical ridge of the limb bud; yet, terminal structures of the wing (and leg) developed in more than 90% of the grafts. Conversely, when the proximal portion of the pre-axial material was grafted to the dorsal aspect of the wing (or hind-limb) bud, but near to its base, or to the somite region, formation of digits did not occur.

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Emb. 2472, 12 day. Ecto- and mesoderm from the pre-axial border of the wing and hind-limb anlage (St. $23^1/_2$) grafted to the distal third of the same limb buds (cf. *inset*). Digits (*arrows*) developed from the graft.

Under the conditions of the present experiments, the formation of terminal parts of the limb from mesenchymal territories which do not give rise to fingers in the normal development should depend on influences exerted by the distal mesodermal districts of the host limb bud upon the grafted pre-axial mesenchyme, as previously suggested by other experiments (Amprino and Camosso[3]).

Résumé. De nouvelles données expérimentales semblent confirmer l'hypothèse que chez l'embryon de poulet le développement des extrémités peut s'effectuer indépendamment de l'influence inductrice de l'épaississement apical de l'ectoderme.

R. Amprino and M. Camosso

Istituto di Anatomia umana, Università di Bari (Italy), August 1, 1960.

PRO EXPERIMENTIS

L'enregistrement microphotographique des courants d'eau autour d'un cilié

L'observation des courants d'eau provoqués par les cils des infusoires ciliés est d'une importance remarquable dans les études du fonctionnement de l'appareil ciliaire et de la cinétique générale du mouvement ainsi que dans les recherches sur l'absorption des particules alimentaires. Il n'existe à présent qu'une méthode d'analyse de ces courants – l'observation immédiate et le dessin à main du mouvement des particules suspendues dans le milieu liquide agité par le cilié. Cette technique était employée par plusieurs chercheurs, le plus souvent dans le cas des paramécies introduites dans une suspension de l'encre de Chine[1].

C'est la méthode d'enregistrement macrophotographique des trajectoires des paramécies mêmes[2], qui inspire à chercher un procédé microphotographique donnant l'image exacte des courants d'eau dus au battement ciliaire. L'idée essentielle se réduit au fait qu'une particule bien éclairée se déplaçant dans l'espace obscure peut marquer la trace de son trajet sur la pellicule photographique pendant une exposition assez longue.

Pour obtenir le champ sombre on a employé un condensateur conique à fond noir relié à la face inférieure du porte-objet par l'huile de cèdre. Le miroir du microscope était éclairé par une lampe très forte (15000 Lux sur le miroir). Les particules suspendues qui font marquer les microcourants d'eau doivent bien réfléchir la lumière et conserver les dimensions assez homogènes de quelques microns de diamètre. Ce sont les émulsions des graisses en général et le lait en particulier, qui répondent bien aux conditions données. Le lait ordinaire qui est nocif pour les protozoaires, dilué 1:10 est déjà convenable dans le cas de *Paramecium caudatum* et de *Stentor coeruleus* employés dans cette étude. Les poses étaient prises sur les pellicules Agfa Isopan-ISS de sensibilité 21° DIN (100 ASA), l'exposition variant de 1–3 sec. L'agrandissement 280 × (l'objectif 40 ×) donnait les meilleurs résultats.

Les cils d'une paramécie arrêtée contre une fibre de coton ne cessent pas de travailler et ils produisent un grand tourbillon le long du corps, les cils antérieurs amenant l'eau du devant de l'animal (Fig. 1). Les particules d'émulsion, qui se trouvent tout près de la surface ciliée de la paramécie présentent souvent un mouvement sinusoïdal dont le trajet correspond par son allure au métachronisme du mouvement ciliaire (Fig. 2). Quant aux battements des cils dans la région péristomale de la paramécie, la méthode d'enregistrement microphotograopique met nettement en évidence leur rôle et la manière d'agir dans l'attraction et l'absorption de la nourriture (Fig. 3). Pour arriver à des résultats irréprochables on peut faire passer les paramécies 48 h avant l'enregistrement par une solution de 5 *mM* d'hydrate de chlorale, qui détruit les cils locomoteurs sans toucher les cils péristomaux[3]. Dans le cas de *Stentor coeruleus* les courants alimentaires peuvent également être enregistrés en dépit du fait que l'animal même, qui se tient debout au-dessous du niveau photographié, reste invisible (Fig. 4).

L'enregistrement des courants d'eau autour d'un cilié qui se déplace est beaucoup plus difficile que dans le cas d'un cilié immobile. Pendant les quelques secondes nécessaires pour photographier les courants, l'animal change considérablement sa position, donc le propre mouvement de tout son corps efface complètement l'image délicate des trajets suivis par les particules de l'émulsion. L'immobilisation de la paramécie obtenue par des changements de la viscosité du milieu ne donne aucun résultat, étant donné que les substances colloïdes introduites dispersent la lumière, détruisant l'effet du champ noir. Il ne reste qu'à ralentir la locomotion en travaillant dans une chambre froide ($t = +\ 2°C$) et à enregistrer dans ces conditions

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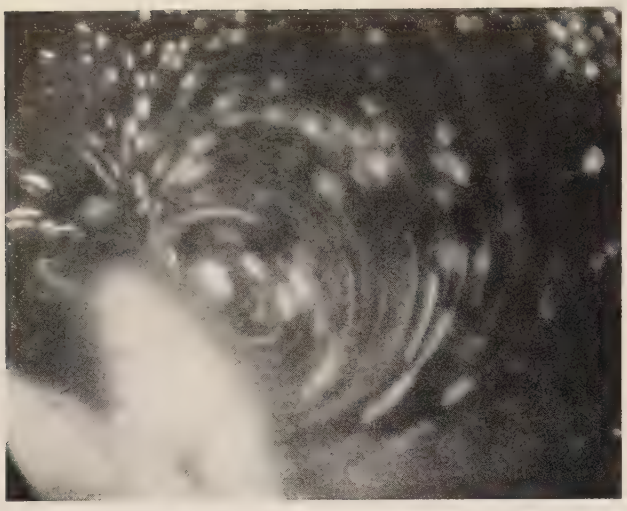

Fig. 1

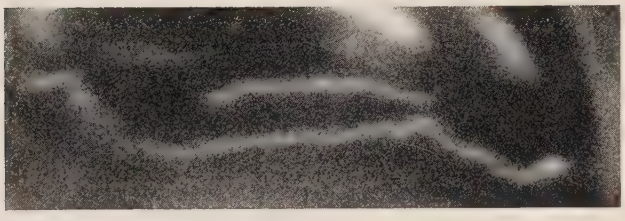

Fig. 2

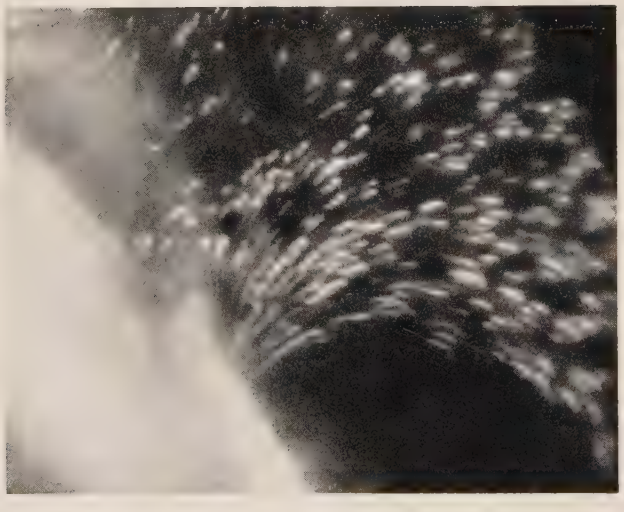

Fig. 3

les mouvements d'eau entourant la partie antérieure de l'animal qui entre dans le champ d'observation, ou les courants laissés en arrière par une paramécie qui est en train de sortir du cadre.

Les premières observations indiquent que la méthode d'enregistrement microphotographique est également utilisable dans le cas d'autres êtres unicellulaires munies de cils ou de flagelles. En comparaison avec la méthode classique du dessin, elle élimine l'influence d'une impression subjective du chercheur, elle permet d'analyser toute l'image compliquée simultanément dans tous ses détails, et enfin elle semble remplacer une illustration par des documents qu'on peut obtenir en nombre voulu.

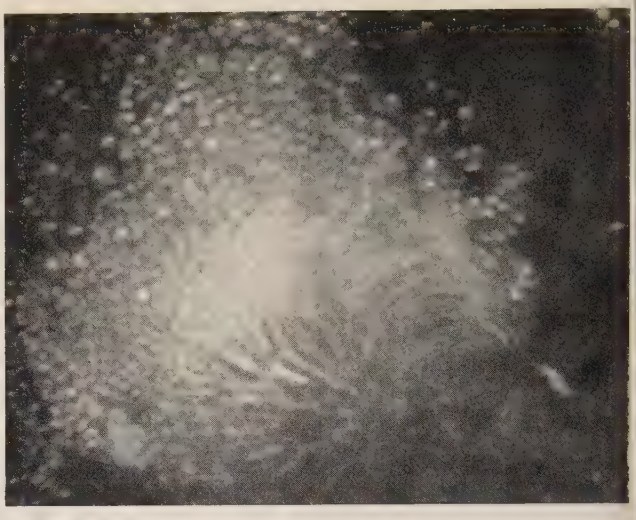

Fig. 4

Summary. The movement of a liquid medium around a protozoan cell evoked by cilia was registered microphotographically. Bright illuminated particles of emulsion or suspension, photographed in the dark field during a long exposure time, mark a course of the water microstreams.

A. GREBECKI

Institut Nencki de la Biologie expérimentale, Varsovie (Pologne), le 1er octobre 1960.

PRO EXPERIMENTIS

Further Simplification of a Supernatant Flow System for Tissue Culture

This paper concerns a further simplification of flow systems for automatic uniform provision of small volumes of supernatant to tissues in culture previously described from this laboratory[1,2]. The system is illustrated in the Figure.

The supernatant flows from a 15 mlcapacity Buchner funnel with medium porosityfritted disc into the culture chamber B and thence to the collection bottle C. The rate of supernatant flow is regulated automatically by the unaided passage of air through the dialysing membrane D. The piece of dialysing membrane is held in an airtight fashion over the end of a length of Pyrex Nr. 234100 glass tubing by a segment of latex tubing ($^1/_4$" I.D., $^1/_{16}$" wall). (The rate of flow can be altered by using glass tubing of different diameters or membranes of different porosity). The open end of the glass tubing is inserted into the upper end of the Buchner funnel through a red rubber, sleevetype, serum stopper. The fritted disc prevents the regurgitation of air into the reservoir from the culture chamber and thus helps smooth the flow of supernatant. When the flow system and chamber is first put into the incubator, the expansion of air in the Buchner funnel forces 3 or 4 ml of supernatant through instead of the usual daily 1–2 ml.

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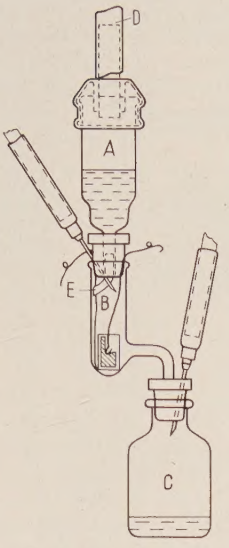
This has the beneficial effect of helping to wash away blood or breakdown products resulting from the explantation. The needle air-vents with cotton bacterial filters in the culture chambe B and receiving bottle C allow free exit for gases to the external air and promote free flow from B to C. The piece of cellulose sponge E inserted into the lower end of the Buchner funnel and touching the side of the culture chamber serves to conduct the supernatant to the side of the culture tube, smooth out the flow and prevent traumatisation of the culture by drops of supernatant. A piece of chemically clean braided fiberglass sleeving in the outflow arm of the culture tube facilitates the exit of supernatant from the culture tube and thus helps to prevent changes in the level of supernatant in the culture chamber. Bacteria are filtered out of the air during its passage through the diaphysing membrane thus preservin gthe sterility of the supernatant.



Schematic diagram of apparatus

Zusammenfassung. In der Arbeit wird eine Einrichtung für kontinuierliche Flüssigkeitszufuhr beschrieben, wie sie für Gewebskulturen wertvoll sein kann. Durch eine feinporöse Membran kann die Menge der Flüssigkeit reguliert werden.

A. W. B. Cunningham and N. O. Lunell[3]

Tissue Dynamics Laboratory, Pathology Department, University of Texas Medical Branch, Galveston (Texas), November 21, 1960.

COGITATIONES

Spontaneous Miniature Activity and Gradation of Transmission at the Neuromuscular Junction

The discovery of the randomized spontaneous miniature activity at the neuromuscular junction by Fatt and Katz[1,2] opened new perspectives for the interpretation of facilitation of synaptic transmission[3–5]. Specifically, it was shown in frog- and mammalian nerve-muscle preparations that the neurally evoked endplate potential (epp) results from a statistical coincidence of quantal units of identical magnitude (i.e. the min epp's)[6–8]; that a conditioning nerve impulse enhances the probability of occurrence of spontaneous miniature activity as well as increases the quantum content of response to a subsequent nerve volley[9,10]; and that the quantum content of a neurally evoked response increases in the course of tetanic stimulation[9,10], while marked augmentation of spontaneous miniature activity persists for several minutes in the post-tetanic period[8,11].

However, del Castillo and Katz ([12], see there Table I) also pointed out that rate of spontaneous miniature discharges and quantum content of evoked epp are not necessarily covariant: e.g. Mg ions effect a decrease of quantum content of epp, while leaving the frequency of spontaneous miniature activity unaltered. Furthermore, a decrease of quantum content of epp after prolonged tetanic stimulation is accompanied by increase of min epp-frequency[9]. Weak anodic polarization of terminal motor nerve branches augments the evoked epp, but the spontaneous discharge rate remains unaltered; cathodic polarization, on the other hand, depresses transmission and augments spontaneous discharge rate[12,13]. An additional instance of dissociation between spontaneous and evoked activity is provided by the fact that spontaneous discharges continue in the absence of Na-ions and in preparations depolarized by K_2SO_4[14], wherefrom it is apparent that spontaneous miniature activity 'does not depend upon the occurrence of electrical activity of the action potential type in any structural unit within the nerve terminal'[5].

To these instances of dissociation between direction of change of spontaneous and evoked junctional activity may now be added the finding of Kraatz and Trautwein[15] that 2,4-dinitrophenol, while greatly augmenting spontaneous random discharge rate, diminishes the epp-amplitude. Hydrazinium-ions[16], and tetrodotoxin in the presence of NH_4^+-ions[17] block transmission without affecting the discharge rate of min epp's. Conversely, guanidine apparently augments the evoked epp[18], while spontaneous miniature activity does not change[17]; however, spontaneous giant potentials appear with an amplitude smaller than that of evoked epp's, but larger than that of min epp's. Botulinus toxin, on the other hand, depresses frequency of spontaneous discharges and reduces the amplitude of the evoked epp as well[11].

In summary, then, there is ample evidence that the mechanism for spontaneous quantal discharges can operate in autonomy from nervous control; and that the frequency of spontaneous activity does not uniquely define the potential of the presynaptic apparatus to transmit incoming nerve volleys to the postsynaptic site. The differences between spontaneous and evoked junctional activity, to which del Castillo and Katz[3] directed attention, can, however, be ascribed to the responsiveness of the motor nerve endings to invasion by a nerve impulse (see: factor 'N' in [12]). This, then, would constitute an additional limiting step in the chain of events of transmission, and would attribute to spontaneous miniature activity a permissive, rather than a determining role in junctional transmission and facilitation of transmission.

It then becomes pertinent to inquire of what nature this limiting neural event is, and whether it is subject to gradation by physiologic and pharmacologic parameters.

In the spinal cord, posttetanic potentiation of the monosynaptic reflex is paralleled by augmentation of the pre-

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synaptic action potential amplitude[20]. This increase of action potential height is attributed to posttetanic hyperpolarization of presynaptic nerve fibres[20–22]. Since terminal anelectrotonus must also result in hyperpolarization of presynaptic neural structures, DEL CASTILLO and KATZ[12] suggested that anelectronic facilitation of neuromuscular transmission is, likewise, related to increase of amplitude (and possibly duration) of the action potential in terminals (see also [23]). Facilitation of neuromuscular transmission by conditioning nerve stimulation can, accordingly, be attributed to invasion of motor nerve terminals by the test volley during the positive afterpotential left by the preceding nerve impulses.

Posttetanic facilitation of transmission at the mammalian neuromuscular junction is accompanied by the occurrence of retrograde motor nerve activity: specifically, tetanic stimulation with ensuing transmission failure (presumably due to presynaptic conduction block[24,25]) is followed by a period during which single nerve impulses elicit repetitive activity in muscle fibres, and antidromically conducted trains of impulses in corresponding motor nerve fibres[26,27]. These posttetanic effects are identical with the effects obtained on single (or double) volley stimulation after administration of agents classed 'facilitators' of neuromuscular transmission: a single orthodromic volley is, then, also seen to elicit repetitive activity in muscle fibres, and to set up a train of repetitive antidromic impulses in the motor axon which carried the primary efferent volley[28–33]. When these repetitive antidromic responses are genuine (i.e.: originating in the motor unit whose axon conducted the primary efferent volley[34]), they are followed by a characteristic recovery cycle[27]: a second efferent motor nerve impulse following the conditioning stimulus within 1 to about 4 msec greatly augments repetitive muscle- and antidromic nerve activity, while a test stimulus at 50 to about 100 msec does not elicit repetitive activity in either muscle or nerve. This time course of augmentation and depression of repetitiveness coincides with that of the sequence of negative and positive afterpotentials in nerve fibres with slow conduction velocity; the early phase of augmentation corresponding to negative, and the late phase of depression corresponding to positive afterpotentials. The generation of antidromic repetitive activity was attributed to a negative afterpotential in motor nerve terminals[27]. A prolongation of the negative afterpotential in frog motor nerve terminals in the presence of tetraethylammonium ions was observed by KOKETSU[35]. The possibility that the negative afterpotential in motor nerve terminals constitutes *per se* a transmitter controlling agency, was explored by KUFFLER[36]: since veratrine prolongs nerve afterpotentials without prolonging the active phase of the transmitter process, this possibility can be excluded[37].

On the basis of these considerations, it appears more appropriate to view the magnitude of the terminal membrane breakdown on orthodromic invasion as the critical event in the neural control of transmission[20,22]. Provided augmentation of afterpotentials reflects hyperpolarization (equivalent to an anelectronic state) of the terminal nerve membrane[38], an increase of the terminal action potential can be inferred from events which reflect augmentation of the terminal afterpotentials, i.e. postactivity motor nerve repetition[27].

It then follows that this hyperpolarized state of motor nerve terminals can be brought about by tetanic stimulation alone, and by single or repetitive nerve shocks in the presence of a variety of quaternary ammonium ions with, and without[33], cholinesterase inhibiting activity; agents whose minimal structural requirements coincide with those of agents which increase the polarizability of nerve membranes ([39]; for review, see[40]). Furthermore, it becomes permissible to identify the phasic changes of augmented afterpotentials with the oscillatory changes of nerve membrane potential constituting the 'nerve reaction' described by LORENTE DE NÓ[41]. It is, therefore, proposed that genuine postactivity motor nerve repetition whose initiation and recovery cycle can be attributed to negative and positive afterpotentials in nerve terminals, reflects an anelectronic state of these terminals and, accordingly, indicates their facilitator potentiality[42].

From the context of these findings, it emerges that transmission at the neuromuscular junction, while operating through the quantal mechanism, is subject to gradation by the terminal electrical event whose absolute magnitude depends on the membrane characteristics of motor nerve terminals. Conversely, the transmitter- and facilitator potentiality of a junction is not uniquely defined by the frequency of spontaneous miniature activity; nor can a neural mechanism of facilitating (or depressant) action be excluded for agents which do not alter the spontaneous discharge rate of min epp's.

Zusammenfassung. Aus den bisher veröffentlichten experimentellen Befunden über presynaptische, für die neuromuskuläre Erregungsübertragung massgeblich erscheinende Vorgänge wird geschlossen, dass die Förderung der Erregungsübertragung in direkter Beziehung zur Grösse des Aktionspotentials in den motorischen Nervenendigungen steht, während die Frequenz der spontanen Miniaturentladungen an sich kein eindeutiges Mass für die Intensität des Übertragungsvorganges von Nervimpulsen zum Muskel darstellt.

G. WERNER

Department of Pharmacology, Cornell University Medical College, New York, November 18, 1960.

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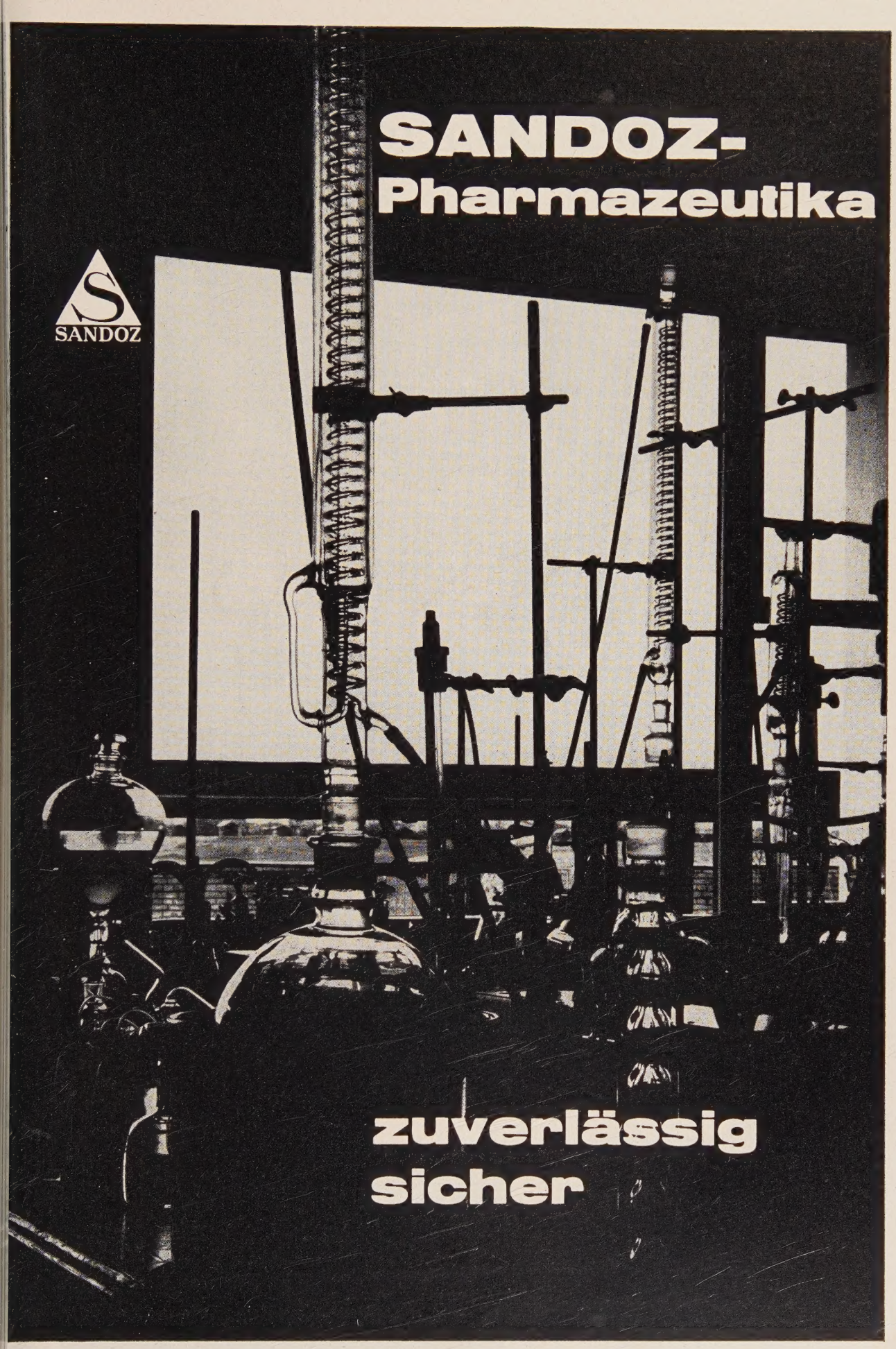
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1er Symposium international de chimiothérapie anti-infectieuse et antimitotique
Erstes Internationales Symposium für antiinfektiöse u. antimitotische Chemotherapie

Genève, 12–13 septembre 1959

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III. Corticosteroids in the Treatment of Infectious Diseases – Les corticostéroïdes dans le traitement des maladies infectieuses – Corticosteroide in der Behandlung von Infektionskrankheiten. Moderator: G. Bickel (Genève).
IV. Chemotherapy of Cancerous Affections – La chimiothérapie des affections cancéreuse – Chemotherapie bei Krebskrankheiten. Moderator: D. A. Karnofsky (New York, N.Y.).
V. Grafting of Bone Marrow in Man – Les essais de greffe de mœlle chez l'homme – Transplantation von Knochenmark beim Menschen. Moderator: J. Bernard (Paris).

Verlangen Sie detaillierte Angaben über die Bände 1–7 — Zu beziehen durch jede Buchhandlung

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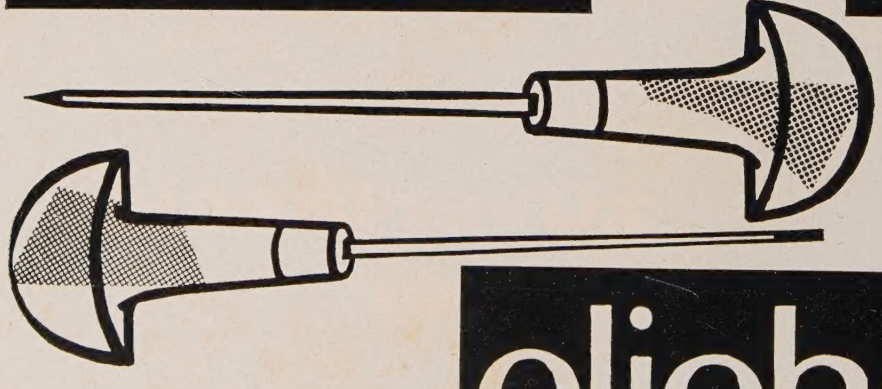

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Basel
Schützenmattstr. 31
Tel. 061/24 99 10/19